

1c837 U.S. PTO

Please type a plus sign (+) inside this box ☐Approved for use through 9/30/00, OMB 0654-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.UTILITY
PATENT APPLICATION
TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. Smart-Biggar

First Inventor or Application Identifier Adrien R. Beaudoin

Title C-8 Substituted Purine Analogs And Their Use As Inhibitors of
Nucleotide Triphosphate Diphosphohydrolases

Express Mail Label No. EK290771439US

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO:

Commissioner for Patents
Box Patent Application
Washington, D.C. 202311 ☒ Fee transmittal Form
(Submit an original and a duplicate for fee processing)2 ☒ Specification [Total 40]
(preferred arrangement set forth below)

- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed Sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 ☒ Drawing(s) (35 USC 113) [Total Sheets 8]

4. Oath or Declaration [Total Pages 3]

a. ☒ Newly executed (original or copy)b. ☐ Copy from prior Application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)

(Note Box 5 below)

i. ☐ DELETION OF INVENTOR(S)
Signed Statement attached deleting
inventor(s) named in prior application,
see 37 CFR 1.63(d)(2) and 1.33(b).5 ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application from
which a copy of the oath or declaration is supplied
under Box 4b, is considered as being part of the
disclosure of the accompanying application and is
hereby incorporated by reference herein.6. ☐ Microfiche Computer Program (Appendix)7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- ☐ Computer readable Copy
- ☐ Paper Copy (identical to computer copy)
- ☐ Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

8 ☐ Assignment Papers (cover sheet & documents)9 ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(where there is an assignee)10 ☐ English Translation Document (if applicable)11 ☐ Information Disclosure
Statement (IDS)/PTO-1449 ☐ Copies of IDS
Citations12 ☐ Preliminary Amendment13 ☒ Return receipt postcard (MPEP 503)
(Should be specifically itemized)14 ☐ *Small Entity Statement filed in prior application
Statement(s) ☐ Status still proper and desired15 ☐ Certified copy of priority Document(s)
(if foreign priority is claimed)16 ☐ Other:* A new statement is required to pay small entity fees, except where
one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application no. _____

Prior application information: Examiner: _____ Group/Art Unit: _____

18. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Labelor ☒ Correspondence address

(Insert Customer No. or Attach bar code label)

NAME

Nicholas J. Seay

Quarles & Brady LLP

ADDRESS

P O Box 2113

CITY

Madison

STATE

WI

ZIP CODE

53701-2113

COUNTRY

US

TELEPHONE

608/251-5000

FAX

608/251-9166

Name (Print/Type)

Nicholas J. Seay

Registration No. (Attorney/Agent)

27,386

Signature

Date

June 9, 2000

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, Washington, DC 20231. QBMA\220492



Firststar Plaza
Post Office Box 2113
Madison, Wisconsin 53701-2113
Tel 608.251.5000
Fax 608.251.9166
www.quarles.com

Attorneys at Law in:
Chicago (Quarles & Brady LLC)
Milwaukee
Naples
Phoenix
West Palm Beach



June 9, 2000

Commissioner for Patents
Box Patent Application
Washington DC 20231

Re: Filing New Patent Application

Dear Sir:

Enclosed for filing please find a new patent application entitled:

C-8 SUBSTITUTED PURINE ANALOGS AND THEIR USE OF INHIBITORS OF
NUCLOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES

by Adrien R. Beaudoin
Fernand-Pierie Gendron
Efrat Halbfinger
Bilha Fischer

The undersigned hereby certifies that this document is being deposited with the United States Postal Service today, June 9, 2000, by the "Express Mail" service, utilizing Express Mail label number EK290771439US, addressed to: Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Please indicate receipt of this application by returning the attached postcard with the official Patent and Trademark Office receipt and serial number stamped thereon.

Respectfully submitted,

Enclosures
QBMAD\220491

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION			Docket No.
Serial No.	Filing Date	Patent No.	Issue Date
Applicant/ ADRIEN R. BEAUDOIN, ET AL Patentee:			
Invention: C-8 SUBSTITUTED PURINE NUCLEOTIDE ANALOGS AND THEIR USE AS INHIBITORS OF TRIPHOSPHATE DIPHOSPHOHYDROLASES			
I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below: NAME OF ORGANIZATION: <u>UNIVERSITÉ DE SHERBROOKE</u> ADDRESS OF ORGANIZATION: <u>Bureau de liaison entreprises-Université</u> <u>Sherbrooke, Quebec</u> <u>J1K 2R1, Canada</u>			
TYPE OF NONPROFIT ORGANIZATION: <input checked="" type="checkbox"/> University or other Institute of Higher Education <input type="checkbox"/> Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3)) <input type="checkbox"/> Nonprofit Scientific or Educational under Statute of State of The United States of America Name of State: Citation of Statute: <input type="checkbox"/> Would Qualify as Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3)) if Located in The United States of America <input type="checkbox"/> Would Qualify as Nonprofit Scientific or Educational under Statute of State of The United States of America if Located in The United States of America Name of State: Citation of Statute:			
I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in: <input checked="" type="checkbox"/> the specification to be filed herewith. <input type="checkbox"/> the application identified above. <input type="checkbox"/> the patent identified above.			
I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).			

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☒ no such person, concern or organization exists.
☐ each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: _____
TITLE IN ORGANIZATION: _____
ADDRESS OF PERSON SIGNING: _____

SIGNATURE: _____ DATE: _____

77473-10

1

C8-SUBSTITUTED PURINE NUCLEOTIDE ANALOGS AND THEIR USE AS INHIBITORS OF NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES

FIELD OF THE INVENTION

5

The present invention relates to C8-substituted purine nucleotide analogs and their use as inhibitors of nucleoside triphosphate diphosphohydrolases (NTPDases), and is particularly concerned with such compounds which provide
10 effective and specific inhibition of NTPDases.

BACKGROUND OF THE INVENTION

In 1971, results of extensive studies on
15 neurotransmission, which was resistant to conventional adrenergic and cholinergic antagonists, led Burnstock to propose that the purine nucleotide ATP and/or the purine nucleoside adenosine, released at synaptic junctions, might mediate a non-adrenergic, non-cholinergic signalling (1-7).
20 Burnstock also hypothesized that nerves released purines which interact on their target cells with purinergic receptors (or purinoceptors) for either ATP, or its breakdown product adenosine (8, 9). The putative ATP-selective receptors were termed P₂-purinoceptors, whereas the adenosine receptors, were
25 termed P₁-purinoceptors (10). Soon, purinoceptors were identified, characterized, and localized in a variety of systems, organs, cells and cell extracts. At the beginning, purinoceptors were classified according to their pharmacological and physiological properties, but with the
30 advent of molecular biology tools, genes encoding purinoreceptors were cloned and a new classification emerged (see 11 for a complete review). Extracellular ATP and ADP and its metabolite adenosine exert multiple effects through these purinoceptors. In the cardiovascular system, these compounds

006090-276560

77473-10

2

influence platelet aggregation, vascular tone, heart function and recruitment of blood cells involved in inflammatory processes (12-15). In the digestive system, it affects electrolyte secretion, gastrointestinal motility, stomach acid
5 secretion and other secretions coming from accessory glands: parotid, liver and exocrine pancreas (16-20). Presence of purinoceptors in the immune system also support a role of extracellular purines and pyrimidines in the immune response (11, 21-25). Presence of these receptors in the central and
10 peripheral nervous systems also supports a role in neurotransmission for these compounds (26-29). These localizations combined with the effects induced by the administration of nucleotides confirm the functions of these nucleotides and their metabolites.

15 A fundamental question is what determines extracellular concentrations of nucleosides and nucleotides in the extracellular compartment. Basically, there are five parameters involved: 1-Rate of release from the source (cell); 2-Rate of diffusion and size of the extracellular compartment;
20 3-Metabolism by ectonucleotidases; 4-Binding to proteins on the cell surface; and 5-Uptake by the cells (translocation or endocytosis). Ectoenzymes with ectonucleotidase activities often localized in proximity of the target cells are believed to play key roles as modulators of the purine or pyrimidine
25 actions. Among the ectoenzymes which display ectonucleotidase activities, one finds alkaline phosphatase [EC 3.6.1.3] which is widely distributed in the different systems of the body, protein kinase reported in certain cell types, ecto-nucleotide pyrophosphatase/phosphohydrolase [EC n.d.] which converts
30 nucleoside triphosphate into nucleoside monophosphate and 5'-nucleotidase [EC 3.1.3.5] which convert nucleoside monophosphate into nucleoside (30-34).

00591177.060900

77473-10

3

Ectonucleotidases, often located on the target cells, rapidly dephosphorylate the nucleotide into the corresponding nucleoside thereby ending the P_2 stimulation and thereby inducing a P_1 type stimulation (31, 33, 36). Quite often, the physiological response elicited by the nucleoside antagonizes the action induced by the corresponding nucleotide (adenosine vs ATP) (14-15). Adenosine is generally considered as a negative feedback modulator (retaliatory metabolite) of cell and organ energy demand and consumption. It interacts with P_1 purinoceptors which comprise at least four subtypes A_1 , A_{2A} , A_{2B} and A_3 , first classified into those that inhibit (A_1) and those that stimulate adenylate cyclase (A_2) (11). They were later classified according to their pharmacological properties and they are now distinguished by their amino-acid sequences (11).

Once released, nucleotides and nucleosides diffuse in the extracellular space and reach their receptor on target cells. Many enzymes contribute to the extracellular metabolism of nucleotides including alkaline phosphatase, ectokinases and deaminases. Perhaps the most important ones are those that convert nucleotides into nucleosides. Many reports have described ecto-ATPase, ecto-ADPase, and ecto-5'-nucleotidase activities in a variety of tissues and cells. The latter was purified, characterized biochemically, and its encoding gene was defined (34). As for the conversion of ATP to ADP and AMP, up until recently, it was believed that two distinct ecto-enzymes were involved in the conversion of ATP to ADP, and ADP to AMP, i.e., ecto-ATPase and ecto-ADPase, respectively (30). The detection of the NTPDase at the surface of vascular cells has presented another alternative for the conversion of ATP to AMP at the cell surface (37). The identification of a mammalian ATP diphosphohydrolase or apyrase goes back to the early 1980s when LeBel et al. described an enzyme that could sequentially catalyse the hydrolysis of γ and β phosphate residues of triphospho- and diphosphonucleosides (38). In a

77473-10

4

series of studies, the enzyme was purified, characterized, and identified as an ectoenzyme (39). A second isoform was identified, purified, and characterized in the bovine aorta (40) and placenta (41). Recent reports describing the homology between potato apyrase and human CD39, showing a comparison of bovine and porcine ATPDases, and the cloning and sequencing of the human ATPDase cDNA and reexpression of the human protein in COS cells, led to the demonstration that ATPDase isoform II and CD39 were the same protein (42-44).

Among many reported inhibitors of NTPDases, one finds analogs of purines, heavy metals, such as Cd^{2+} and Hg^{2+} (44, 46) and molecules belonging to the suramin family, Evans blue and also other types of molecules.

Purine analogs, such as β , γ -MetATP, β , γ -imido-ATP and ADP β S, may be used to inhibit the NTPDase (47). These analogs share a common characteristic, that is they all bear a substituted group on the phosphate chain. Moreover, all these analogs are purinoceptor ligands. Other nucleotide analogs have also been reported as NTPDase inhibitors, mainly ARL67156 and PPADS. These analogs have been reported to inhibit ecto-ATPase activity (48-51). Finally, two other purine analogs have been reported as NTPDase inhibitors: fluorosulfonylbenzoyl adenosine (FSBA) and 2-thioether-AMP-S (46, 52). However, contrary to purine analogs, FSBA causes an irreversible NTPDase inhibition.

Many P2 antagonists related to suramin (53), reactive blue (54), reactive red (55), Evans blue (56), trypan blue (56) and small aromatic isothiocyano-sulphonates (57), have been reported to be ecto-nucleotidase inhibitors. Other molecules have been proposed as non-specific NTPDase inhibitors, such as sodium azide, sodium fluoride (46) and 9-amino-1, 2, 3, 4-tetrahydroacridine or THA (58).

Based on the facts that (a) NTPDases play a major role in the regulation of purine nucleotide and nucleoside

77473-10

5

levels and (b) purine nucleotides and nucleosides are involved in and influence a number of biological processes, modulation of the activity of NTDPases may have significant effects on such biological processes. Therefore, there exists a need for effective inhibitors of NTDPases, to better modulate the activity of NTDPases, thus modulating the levels of purine nucleotides and nucleosides, which in turn results in the modulation of a variety of biological processes.

10

SUMMARY OF THE INVENTION

An aspect of the present invention is a C8-substituted purine nucleotide analog, wherein the analog is substituted at the C8 position with a substituent other than H.

15

A further aspect of the present invention is a composition comprising the above-mentioned analog in admixture with a suitable diluent or carrier.

Yet a further aspect of the present invention is a method for modulating the activity of an NTPDase enzyme comprising exposing the enzyme to the above-mentioned analog or composition.

In a preferred embodiment, the present invention provides a method for inhibiting the activity of an NTPDase enzyme comprising exposing the enzyme to the above-mentioned analog or composition.

Yet a further aspect of the present invention is a method for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system, comprising the step of introducing into said system the above-mentioned analog or composition.

Yet a further aspect of the present invention is a method for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or

77473-10

6

metabolite(s) or derivative(s) thereof in said system, comprising the step of introducing into said system the above-mentioned analog or composition.

Yet a further aspect of the present invention is a use of the above-mentioned analog or composition for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system.

Yet a further aspect of the present invention is a use of the above-mentioned analog or composition for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system.

Yet a further aspect of the present invention is a commercial package containing the above-mentioned analog or composition together with instructions for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system.

Yet a further aspect of the present invention is a commercial package containing the above-mentioned analog or composition together with instructions for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in said system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Structures of different ATP analogs.

Figure 2: Scheme of synthesis of 8-thioether-ATP derivatives. A. 10 eq. NaSH/wet DMF/100°C/overnight/100% yield. B. 1. compound 10/MeOH/0.25 M NaOH/RT, freeze drying. 2. alkyl

77473-10

7

bromide/DMF/60°C/overnight/83-93% yield. C. 1. POCl_3 /proton sponge. 2. $\text{P}_2\text{O}_5\cdot\text{H}_2(\text{Bu}_3\text{NH}^+)_2$. 3. 0.2 M TEAB.

Figure 3: Hydrolysis of ATP and analogs by NTPDase.

5 ATP and analogs were used at a concentration of 100 μM . Hydrolysis was carried out at 37°C for 7 min in the presence of 1.9 μg of protein. C2 substituted analogs (2a-d) are all hydrolyzed by the enzyme whereas C8-substituted analogs (6-8) are more or less resistant to NTPDase hydrolysis. Results are
10 expressed as the mean \pm SEM of n replicate (see figure) carried out in triplicate.

Figure 4: Kinetic parameters of bovine spleen NTPase for ATP, ADP and purine analogs. Experiments were carried out
15 in triplicate and results are expressed as the mean \pm SEM of the best fit obtained with GraFit 4 software (Erithacus, UK). Apparent K_m and V_{max} were estimated from Eadie and Hofstee representation and K_i 's from Dixon plots.

20 Figure 5: Inhibitory effect of 8-BuS-ATP, 6e, on ATP hydrolysis by NTPDase. A) Lineweaver-Burk representation of NTPDase inhibition. ATP (10 to 100 μM) and 8-BuS-ATP: 0 μM : \blacklozenge ; 10 μM : \blacksquare ; 25 μM : \blacktriangle ; 50 μM : \bullet ; and 100 μM : \times . B) Dixon plots of NTPDase inhibition. ATP as substrate: 10 μM (\blacktriangle), 25 μM (\blacksquare)
25 and 50 μM (\blacklozenge) and 8-BuS-ATP concentrations as above. 8-BuS-ATP produce a competitive inhibition with an estimated K_i of 10 μM . Results are expressed as the mean \pm SEM of two experiments each in duplicate.

30 Figure 6: Dixon representations of 8- $\text{CH}_2\text{tBuS-ATP}$, 6b, (panel A) and 8-cycloheptyls-ATP, 6a, (panel B) inhibition. ATP concentration: 50 μM (\blacklozenge) and 100 μM (\blacksquare). Analog concentrations (0 to 100 μM). Both analogs act as mixed type inhibitors with estimated K_i of 45 and 31 μM for 8-tBuCH₂S-ATP,

77473-10

8

6b, and 8-cycloheptylS-ATP, 6a, respectively. Experiments were carried out in triplicate and expressed as the mean \pm SEM.

Figure 7: A) Lineweaver-Burk representation of the hydrolysis of 8-thioethyl-ATP, 6c, by NTPDase. B) Dixon representations of 8-thiohexyl-ATP, 6d, inhibition. ATP concentration: 15 μ M (\blacklozenge) and 50 μ M (\blacksquare). Analog concentrations (0 to 100 μ M). Analog 6d acts as a non-competitive inhibitor with an estimated K_i of 16 μ M. Experiments was carried out in triplicate and expressed as the mean \pm SEM.

Figure 8: Purinergic activity of 8-BuS-ATP, 6e.

A) Effect of 8-BuS-ATP (0.1-1000 pmol) on denuded mesenteric bed of guinea pig. Results are expressed as % of control (control = 37 mm Hg). No variations of perfusion pressure were measured. Results are the mean \pm SEM of at least three experiments.

B) Effect of 8-BuS-ATP on the relaxing effect of ATP on intact mesenteric bed. ATP (0.1-10000 pmol) in the presence of 7 μ M of 8-BuS-ATP (open bars) or in absence (control: closed bars). Results are expressed as % of vasodilation measured from a precontracted vessel (200 μ M of noradrenaline). No significant difference was observed. Results are the mean \pm SEM of at least three experiments.

C) Vascular responses with or without endothelium. Endothelium integrity was tested with 100 pmol of NK-1 (closed bar) and blood vessel responsiveness was evaluated by 3 nmol of NaNP (open bar). Results are expressed as % of vasodilation measured from a precontracted vessels (200 μ M of noradrenaline). In denuded mesentery there is significant response to NK-1. Results are the mean of three experiments or more.

77473-10

9

DESCRIPTION OF THE EMBODIMENTS OF THE INVENTION

The present invention provides C8-substituted purine nucleotide analogs. In a preferred embodiment, such analogs are ATP analogs, examples of which the Applicant has prepared and characterized. Such analogs have a variety of uses, a preferred one of which is the inhibition of NTPDases, which the Applicant has characterized. For this use, the compounds of the invention were found to be effective.

The Applicant has selected and synthesized purine nucleotide analogs, more particularly ATP analogs, specifically compounds 2a-d and 6-8 (illustrated in Figures 1 and 2). Based on the promising hydrolytic stability of compounds 6-8, the Applicant has synthesized a new series of 8-thioether ATP analogs, i.e., compounds 6a-e, and has evaluated their use as NTPDase inhibitors. In view of finding a specific and potent NTPDase inhibitor, the Applicant has examined two series of ATP analogs substituted at positions C2 and C8, respectively.

As described in the Examples below, this evaluation revealed that at least one of the compounds tested was improved with respect to, for example, at least one criterion selected from the following:

1. Resistance to NTPDase hydrolysis.
2. Potency as an NTPDase inhibitor.
3. Nature of the inhibition.
4. K_i value.
5. Effects on P2 (e.g. P2X and P2Y) purinergic receptors.

30

Via the above evaluation, the Applicant has first demonstrated that analogs substituted with electron donating groups at C8 were more resistant to NTPDase hydrolysis than the corresponding C2 substituted analogs. Therefore, an aspect of

77473-10

10

the invention are C8-substituted purine nucleotide analogs, a preferred embodiment of the invention being ATP analogs.

The C8-substituted purine nucleotide analogs of the invention may be substituted at this position, for example, by
5 electron donating groups. Such groups include but are not limited to ethers, thioethers and amines. Examples of ethers, thioethers and amines that are aspects of the invention are those with, for example, alkyl groups. Such alkyl groups may be, for example, cyclic-, branched- and/or n-alkyl groups. An
10 example of a preferred cyclic alkyl group according to the invention is the cycloheptyl (C_7H_{13}) group. An example of a preferred branched alkyl group according to the invention is the 2,2-dimethyl-propyl ($(CH_3)_2CCH_2$) group. Examples of preferred n-alkyl groups according to the invention are those
15 up to 6 carbons in length. A particularly preferred embodiment is an n-butyl ($CH_3(CH_2)_3$) group.

The Applicant has further demonstrated that of the C8-substituted ATP analogs analyzed, all of which were found to possess a degree of resistance to NTPDase hydrolysis, compounds
20 6a, 6b, 6d and 6e were more resistant to hydrolysis by NTPDase than compounds 7, 8 and 6c. The Applicant has further evaluated the C8-substituted ATP analogs compounds 6a, 6b, 6d and 6e of the invention for their potency as NTPDase inhibitors, and has demonstrated that all four of these
25 compounds are good inhibitors. Therefore, compounds 6a, 6b, 6d and 6e are further preferred aspects of the present invention.

Of all the compounds tested, compound 6e was found to be most resistant to hydrolysis by NTPDase, and was further found to exhibit competitive inhibition with a K_i value lower
30 than those measured for compounds 6a, 6b and 6d. Therefore, as noted above, the Applicant notes that compounds 6a, 6b, 6d and 6e are further preferred aspects of the present invention, and compound 6e represents a particularly preferred aspect of the invention.

77473-10

11

The Applicant has further evaluated compound 6a of the invention with respect to any effects on purinoceptors, and found that this compound does not interact with either P2X- or P2Y-purinoceptors. Therefore, the compounds of the invention
5 act as, for example, effective and specific inhibitors of NTPDase.

Therefore, the invention provides C8-substituted purine nucleotide analogs, which may, for example, be used for the modulation of NTPDase activity. In a preferred embodiment,
10 the C8-substituted purine nucleotide analogs of the invention may be used for the inhibition of NTPDase activity. Given the wide variety of biological processes which are affected by the purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives, the compounds of the invention may be
15 utilized to alter the activity of such processes via the alteration of the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives.

Accordingly, an aspect of the present invention is a method to alter or modulate the level of purine nucleotides
20 and/or nucleosides and/or their metabolites and/or derivatives in a biological system. A further aspect of the present invention is a method of altering the activity of a biological process which is affected by the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives
25 in a biological system. The modulation of such processes occurs by, for example, the action of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives on cell surface receptors, such as purinoceptors. Such cell surface receptors can act to modulate a large number of biological
30 processes using a variety of mechanisms. Examples of such mechanisms include acting through G-proteins to generate a variety of signalling cascades (e.g., involving inositol phospholipid or other messengers and/or the mobilization of calcium stores), the activation of ligand-gated ion channels,

006099" 050900

77473-10

12

the induction of channels and/or pores, and the modulation of ion fluxes and other responses.

The processes noted above which may be modulated as a result of the modulation of the levels of purine nucleotides and/or nucleosides and/or their metabolites and/or derivatives include, but are not limited to, the following:

1. In the cardiovascular system, such processes include, for example, platelet aggregation; modulation of vascular tone and function (e.g., vasoconstriction and vasodilation) and blood flow; heart function and performance; and the recruitment and adhesion of blood cells involved in inflammatory processes.

2. In the nervous system (central and peripheral), such processes include, for example, neurotransmission.

3. In sensory systems, such processes include, for example, activity of sensory organs and/or cells.

4. In muscle tissues, such processes include, for example, activity (e.g., contractile responses) of visceral smooth muscle and skeletal muscle.

5. In the pulmonary system, such processes include, for example, secretion by cells of the pulmonary system.

6. In the immune system, such processes include, for example, function of various immune cell types, and the modulation of diverse responses of the immune system.

7. In endocrine, neurocrine and exocrine systems, such processes include, for example, secretion of a variety of compounds from a variety of cell types in these systems. Such cell types include, for example, those present in the pancreas, and parotid, lacrimal, thyroid, adrenal and pituitary glands.

8. In paracrine cells, such as platelet and mast cells, such processes include, for example, secretion of compounds from these cells. Further, as noted above, the processes affected also include platelet aggregation, and

006090" 22T6960

77473-10

13

thrombus formation, as well as the additional recruitment of new platelets to the developing thrombus.

9. In the reproductive system and cells of the reproductive system, such processes include, for example, contraction of smooth muscle tissue (e.g., myometrium) and activity of germ cells.

10. In hepatic tissue, such processes include, for example, hepatic tissue function and the biochemical and biological processes which occur therein (e.g. gluconeogenesis and glycogenolysis), as well as the secretion of compounds (e.g., thromboxanes and prostaglandins) from hepatic cells and tissue.

11. In renal and gastrointestinal tissues, such processes include, for example, secretion (e.g., of electrolyte(s) and stomach acid) from such tissues and cells therein, and gastrointestinal motility.

12. In connective tissue, skin and bone, such processes include, for example, the modulation of a variety of activities and functions within these tissues and cells therein, such as growth and differentiation.

13. In tumor cells, such processes include, for example, a variety of activities and functions, such as growth regulation. Therefore, the growth of tumor or cancer cells and tissue may be modulated, and thus the invention further provides compounds, compositions, methods, uses, and commercial packages for the treatment of cancer.

Therefore, the invention provides compounds, compositions, methods, uses, and commercial packages for the modulation of activities and function in the cardiovascular, nervous, immune, inflammatory, sensory and reproductive systems; in muscle, endocrine, neuroendocrine, exocrine, paracrine, germ, hepatic, renal and gastrointestinal cells and tissues; as well as in connective tissue, skin and bone. The invention further provides compounds, compositions, methods,

005090" 4476550

77473-10

14

uses, and commercial packages for the modulation of a process such as aggregation and thrombogenicity. In an embodiment, such modulation comprises an increase in aggregation and thrombogenicity.

5 Yet a further aspect of the present invention is the use of a compound of the invention for, for example, the methods and purposes described above.

The compounds of the present invention may also be adapted for certain applications using methods known in the
10 art. For example, a compound of the invention may be attached to a solid phase or matrix. In this form, the compound may be utilized for the isolation and purification of species with which it binds/interacts, for example, using the technique of affinity chromatography. Such species comprise proteins which
15 bind purine nucleotides and/or their metabolites and/or derivatives, an example of such a protein being an NTPDase enzyme. In other embodiments, the compounds of the invention may be modified for their use in a variety of methods such as diagnostic methods.

20 The compounds of the invention or corresponding modified versions may be a component of an appropriate composition of the invention, comprising a compound of the invention or a corresponding modified version and a suitable diluent or carrier. Such compositions may be, for example,
25 utilized in the uses and methods described above. Such compositions include pharmaceutical compositions, comprising a compound of the invention or a corresponding modified version and a suitable pharmaceutically acceptable diluent or carrier. The compounds, corresponding modified versions, or the
30 compositions of the invention may also be a component of a commercial package of the invention, which comprises a compound or composition of the invention together with instructions for, for example, the uses and methods described above.

0059117-060900

77473-10

15

The following examples are provided in order to illustrate the embodiments of the present invention and are not meant to limit the scope of the invention.

5 **Example 1: Synthesis, purification and characterization of ATP analogs.**

General methods. New compounds were characterized by proton and carbon nuclear magnetic resonance using a Bruker AC-200 or DPX-300 spectrometer. The chemical shifts are reported
10 in ppm relative to TMS as an internal standard. Nucleotides were characterized also by ^{31}P -NMR in D_2O using 85% H_3PO_4 as an external reference on a Bruker AC-200 spectrometer. Mass spectra were recorded on an AutoSpec-E-FISION VG high resolution Mass Spectrometer. Nucleotides were characterized
15 by FAB (fast atom bombardment) and high resolution FAB using a glycerol matrix under FAB negative conditions on AutoSpec-E-FISION VG high resolution Mass Spectrometer. Separation of the newly synthesized nucleotides was achieved using LC (Isco UA-6) using DEAE A-25 Sephadex (HCO_3^- form) anion exchanger as
20 described below. Final purification was done using an HPLC (Merck-Hitachi) system using a semi-preparative LiChroCART LiChrospher 60 RP-select B column (1 x 25 cm, Merck KGaA) and a linear gradient of 0.1 M triethylammonium acetate buffer (TEAA, pH 7.5) and methanol (see below) at 6 mL/min flow rate. For
25 analytical purposes, a LiChroCART LiChrospher 60 RP-select B column (250 mm x 4.6 mm, Merck KGaA) was used applying the same gradient as above at 1 mL/min flow rate. The purity of the nucleotides described below was evaluated on an analytical column in two different solvent systems. One solvent system
30 (I) was 0.1 TEAA/ CH_3OH , 80:20 to 20:80 in 20 min. The second solvent system (II) was (A) 5 mM tetrabutylammonium phosphate (TBAP) in methanol and (B) 60 mM ammonium phosphate and 5 mM

0059117-060900

77473-10

16

TBAP in 90% water/10% methanol, applying a gradient of 25% A to 75% A in 20 min.

The selection and synthesis of ATP analogs, compounds 2a-d and 6-8 (Figures 1 and 2), for their evaluation as NTPDase inhibitors were based on the promising hydrolytic stability of compounds 6-8. The desired derivatives, 6a-e, were obtained in three steps from 8-Br-adenosine in good yields (Figure 2). 8-Mercapto-adenosine, 10, was obtained in a quantitative yield from 8-Br-adenosine upon treatment with 10 eq of NaSH in wet DMF at 100°C overnight. The corresponding dry sodium thiolate salt, obtained upon dissolution of 10 in MeOH/0.25 M NaOH and subsequent freeze drying, was treated with the appropriate alkyl bromide in DMF at 60°C to yield compounds 11 in high yields. Finally these compounds were 5'-triphosphorylated, to give nucleotides 6 in reasonable yields (64). Compound 6 of Figure 1E and compound 6e of Figure 2 are one in the same, i.e., with a thiobutyl ($\text{CH}_3(\text{CH}_2)_3\text{S}$) group as the C8 substituent. This compound was prepared as described previously (64).

20 **8-Mercaptoadenosine (10).** NaSH (0.8 g, 10 eq) was added to a solution of 8-bromoadenosine (0.5 g, 1.44 mmol) in DMF (7 mL). The mixture was warmed to 100°C and a few drops of water were added to improve solubility. The mixture was stirred at 100°C overnight. The solvent was evaporated under high vacuum and the residue was coevaporated repeatedly with MeOH, until the residue turned into a solid. The residue was dissolved in water and neutralized with NaOH. After freeze drying, the product was purified on a silica gel column ($\text{CHCl}_3:\text{MeOH}$ 10:1). The product was obtained as a yellowish powder (100% yield, mp 169-170 °C). $^1\text{H-NMR}$ (CD_3OD , 200 MHz) 8.09 (s, 1H, H-2), 6.65 (d, $J = 7$ Hz, 1H, H-1'), 5.01 (dd, $J = 7, 5.5$ Hz, 1H, H-2'), 4.39 (dd, $J = 5.5, 2.5$ Hz, 1H, H-3'), 4.13 (q, $J = 2.5$ Hz, 1H, H-4'), 3.87 (dd, $J = 12.5, 2.5$ Hz, 1H, H-5'), 3.71

77473-10

17

(dd, $J = 12.5$, 3 Hz, 1H, H-5'); ^{13}C -NMR (CD_3OD , 300 MHz) δ 167.88 (C-6), 151.92 (C-2), 148.12 (C-4), 147.88 (C-8), 107.00 (C-5), 88.62 (C-1'), 85.59 (C-4'), 70.70 (C-2'), 70.62 (C-3'), 62.13 (C-5'); MS (CI/ NH_3): m/z 317 $\text{M}+\text{NH}_4^+$.

5

8-(Thiocycloheptyl) adenosine (11a). A suspension of 8-mercaptoadenosine (75 mg, 0.25 mmol) in MeOH (2 mL) was dissolved in 0.25 M NaOH (1 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze drying, the thiolate sodium salt obtained as a yellowish solid, was dissolved in dry DMF (2 mL) and bromocycloheptane (38 μL , 1.1 eq) was added. The solution was stirred overnight under nitrogen at 60°C. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was triturated with petroleum ether/ether 1:1, and then separated on a silica gel column (CHCl_3 :MeOH 20:1). Product 11a was obtained as a white solid in 83 % yield (82 mg) after evaporation and drying, mp 205-6 °C. ^1H -NMR ($\text{DMSO}-d_6$, 200 MHz): 8.07 (s, 1H, H-2), 7.36 (br.s, 2H, NH_2), 5.84 (d, $J = 7\text{Hz}$, 1H, H-1'), 5.00 (dd, $J = 7$, 5 Hz, 1H, H-2'), 4.16 (dd, $J = 5$, 2 Hz, 1H, H-3'), 4.07-3.91 (m, 2H, H-4' & SCH), 3.68 (dd, $J = 12$, 4 Hz, 1H, H-5'), 3.51 (dd, $J = 12.5$, 4 Hz, 1H, H-5'), 2.19-1.93 (m, 2H), 1.84-1.42 (m, 11H); ^{13}C -NMR ($\text{DMSO}-d_6$, 300 MHz): δ 154.80 (C-6), 151.51 (C-2), 150.03 (C-4), 147.94 (C-8), 119.75 (C-5), 88.96 (C-1'), 86.64 (C-4'), 71.31 (C-2'), 71.08 (C-3'), 62.27 (C-5'), 48.42 (SCH), 34.51 (CH_2), 34.14 (CH_2), 27.77 (CH_2), 27.76 (CH_2), 25.14 (CH_2), 25.04 (CH_2). FAB(positive): m/z 396 MH^+ .

30

8-(Thio-2,2-dimethyl-propyl)-adenosine (11b). The compound was prepared as described for 8-(thiocycloheptyl)-adenosine and obtained in 79 % yield (73 mg) as a yellowish

77473-10

18

solid, mp 141-2°C. ¹H-NMR (CD₃OD, 200 MHz) δ 8.06 (s, 1H, H-2), 6.02 (d, J = 7 Hz, 1H, H-1'), 4.99 (dd, J = 7, 5 Hz, 1H, H-2'), 4.33 (dd, J = 5, 2 Hz, 1H, H-3'), 4.17 ("q", J = 2 Hz, 1H, H-4'), 3.88 (dd, J = 12.5, 2.5 Hz, 1H, H-5'), 3.72 (dd, J = 12.5, 3 Hz, 1H, H-5'), 3.42 and 3.35 (AB, J = 7 Hz, 2H, SCH), 1.09 (s, 9H, SCH₂(CH₃)₃); ¹³C-NMR (CD₃OD, 300 MHz) 155.92 (C-6), 152.91 (C-4), 152.11 (C-2), 151.69 (C-8), 110.64 (C-5), 91.19 (C-1'), 88.89 (C-4'), 74.13 (C-2'), 73.20 (C-3'), 64.17 (C-5'), 47.69 (SCH₂), 29.07 (3C, SCH₂(CH₃)₃); MS (CI/NH₃) m/z: 368 (M-H)⁺.

10

8-(Thioethyl)-adenosine (11c). A suspension of 8-mercaptoadenosine (270 mg, 0.9 mmol, in 7 mL MeOH) was dissolved in 0.25 M NaOH (3.6 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze drying, the thiolate sodium salt, obtained as a yellowish solid, was dissolved in dry DMF (3 mL) and bromoethane (101 μL, 1.5 eq) was added. The solution was stirred under nitrogen at room temperature for 3 h. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was separated on a silica gel column (CHCl₃:MeOH 15:1). Product 11c was obtained as a white solid in 93 % yield after evaporation and drying (273 mg), mp 176°C. ¹H-NMR (DMSO-d₆, 200 MHz): 8.05 (s, 1H, H-2), 7.31 (br. s, 2H, NH₂), 5.76 (d, J = 7 Hz, 1H, H-1'), 5.66 (dd, J = 8.5, 3.5 Hz, 1H, OH-5'), 5.42 (d, J = 6 Hz, 1H, OH-2'), 5.21 (d, J = 4 Hz, 1H, OH-3'), 4.99 (br. q, J = 6 Hz, 1H, H-2'), 4.15 (bs, 1H, H-3'), 3.96 (br. s, 1H, H-4'), 3.68 (dt, J = 12, 3.5 Hz, 1H, H-5'), 3.60-3.44 (m, 1H, H-5'), 3.43-3.14 (m, 1H, SCH₂CH₃), 1.36 (t, J = 7 Hz, 3H, SCH₂CH₃); ¹³C-NMR (DMSO-d₆, 300 MHz): 154.49 (C-6), 151.30 (C-2), 150.40 (C-4), 148.53 (C-8), 119.66 (C-5), 88.86 (C-1'), 86.63 (C-4'), 71.29 (C-2'), 70.01 (C-3'), 62.24 (C-5'), 26.77 (SCH₂CH₃), 14.85 (SCH₂CH₃); MS (CI/CH₄)

77473-10

19

: m/z 328 MH⁺; High resolution MS: calcd for C₁₂H₁₈N₅O₄S
328.1079, Found 328.1069.

8-(Thio-n-hexyl)-adenosine (11d). The compound was
5 prepared as described for 8-(thioethyl)-adenosine and obtained
in 91 % yield (314 mg) as a white solid, mp 169-171°C. ¹H-NMR
(DMSO-d₆, 200 MHz): δ 8.05 (s, 1H, H-2), 7.29 (br.s, 2H, NH₂),
5.77 (d, J = 7 Hz, 1H, H-1'), 5.67 (dd, J = 9, 3.5 Hz, 1H, OH-
5'), 5.42 (d, J = 6 Hz, 1H, OH-2'), 5.21 (d, J = 4 Hz, 1H, OH-
10 3'), 4.99 ("q", J = 6 Hz, 1H, H-2'), 4.15 (br.s, 1H, H-3'), 3.96
(br.s, 1H, H-4'), 3.67 (dt, J = 12, 3.5 Hz, 1H, H-5'), 3.60-3.43
(m, 1H, H-5'), 3.42-3.18 (m, 1H, SCH₂), 1.69 (quint, J = 7 Hz,
2H, SCH₂CH₂), 1.50-1.18 (m, 6H, -CH₂CH₂CH₂CH₃), 0.86 (t, J = 7
Hz, 3H, CH₃); ¹³C-NMR (DMSO-d₆, 300 MHz): 154.55 (C-6), 151.27
15 (C-2), 150.38 (C-4), 148.71 (C-8), 119.61 (C-5), 88.85 (C-1'),
86.63 (C-4'), 71.28 (C-2'), 71.02 (C-3'), 62.23 (C-5'), 32.37
(SCH₂), 30.70 (CH₂), 28.80 (CH₂), 27.71 (CH₂), 21.99 (CH₂), 13.88
(CH₃); MS (CI/CH₄) : m/z 384 MH⁺; High resolution MS: calcd for
C₁₆H₂₆N₅O₄S 384.1705, Found 384.1696.

20

Nucleoside 5'-Triphosphorylation. Nucleosides 11a-d
were 5'-triphosphorylated according to a published procedure
(60).

25 8-(Thiocycloheptyl)-adenosine 5'-triphosphate (6a).
The compound was obtained in 60 % yield (79 mg). Final
separation was achieved on HPLC applying a linear gradient of
TEAA/ CH₃OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 9.53 min.
¹H-NMR (D₂O, 200 MHz): δ 8.17 (s, 1H, H-2), 6.09 (d, J = 6 Hz,
30 1H, H-1'), 5.15 (t, J = 6 Hz, 1H, H-2'), 4.62-4.51 (m, 1H, H-
3'), 4.37-4.14 (m, 3H, H-4' & H-5'), 3.93-3.75 (m, 1H, SCH),
2.15-1.90 (m, 2H), 1.80-1.36 (m, 11H); ³¹P-NMR (D₂O, 200 MHz, pH

005917-060900

77473-10

20

9) δ -5.34 (d), -10.37 (d), -21.32 (t); UV: λ_{\max} 282 nm. HRFAB: calcd for $C_{17}H_{27}N_5O_{13}P_3S$ 634.0539, Found 634.0540; t_R 14.99 min (95 % purity) using solvent system I, 13.19 min (97 % purity) using solvent system II.

5

8-(Thio-2,2-dimethyl-propyl)-adenosine 5'-triphosphate (6b). The compound was obtained in 65 % yield (77 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH_3OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 7.51 min.

10 1H -NMR (D_2O , 200 MHz): δ 8.21 (s, 1H, H-2), 6.13 (d, J = 6 Hz, 1H, H-1'), 5.20 (t, J = 6 Hz, 1H, H-2'), 4.62 (dd, J = 6 Hz, 1H, H-3'), 4.42-4.25 (m, 3H, H-4' & H-5'), 3.29 and 3.35 (ABq, J = 12 Hz, 2H, SCH_2), 1.06 (s, 3H, $SCH_2(CH_3)_3$); ^{31}P -NMR (D_2O , 200 MHz, pH 9) δ -10.23 (d), -10.79 (d), -22.61 (t); UV: λ_{\max} 282
15 nm. HRFAB: calcd for $C_{15}H_{25}N_5O_{13}P_3S$ 608.0382, Found 608.0360; t_R 12.71 min (96 % purity) using solvent system I, 11.31 min (95 % purity) using solvent system II.

8-(Thioethyl)-adenosine 5'-triphosphate (6c). The
20 compound was obtained in 43 % yield (84 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH_3OH 90:10 to 20:80 in 20 min (6 mL/min). t_R 8.11 min. 1H -NMR (D_2O , 200 MHz): δ 8.15 (s, 1H, H-2), 6.10 (d, J = 6.5 Hz, 1H, H-1'), 5.17 (t, J = 6.5 Hz, 1H, H-2'), 4.65-4.55 (m, 1H, H-3'), 4.40-
25 4.16 (m, 3H, H-4' & H-5'), 3.30 and 3.26 (ABq of t, J = 11.5, 7 Hz, 1H each, SCH_2), 1.39 (t, J = 7 Hz, 3H, CH_3); ^{31}P -NMR (D_2O , 200 MHz, pH 9) δ -5.12 (d), -10.31 (d), -20.98 (t); UV: max 282 nm. HRFAB: calcd for $C_{12}H_{18}N_5O_{13}NaP_3S$ 587.9732, Found 587.9650; t_R 7.16 min (96 % purity) using solvent system I, 3.55 min (94 %
30 purity) using solvent system II.

006090" 42T6560

77473-10

21

8-(Thio-n-hexyl)-adenosine 5'-triphosphate (6d). The compound was obtained in 58 % yield (111 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ CH₃OH 70:30 to 20:80 in 20 min (6 mL/min). t_R 10.73 min. ¹H-NMR (D₂O, 200 MHz): 8.17 (s, 1H, H-2), 6.01 (d, J = 6.5 Hz, 1H, H-1'), 5.19 (t, J = 6.5 Hz, 1H, H-2'), 4.64-4.55 (m, 1H, H-3'), 4.40-4.14 (m, 3H, H-4' & H-5'), 3.32 and 3.24 (ABq of t, J = 14, 7 Hz, 1H each, SCH₂), 1.73 ("quint", J = 7 Hz, 2H, SCH₂CH₂), 1.52-1.09 (m, 6H), 0.82 (t, J = 7 Hz, 3H, CH₃); ³¹P-NMR (D₂O, 200 MHz, pH 9) -5.12 (d), -10.25 (d), -21.03 (t); UV: max 282 nm; t_R 15.25 min (>97 % purity) using solvent system I, 15.52 min (>97 % purity) using solvent system II.

Example 2: Enzymology of purine nucleotide analogs

15

The demonstration of the inhibitory properties of a given compound on a particular enzyme requires that a single site of catalysis is present in the medium. In other words, if a second enzyme competes for the substrate or generates the same reaction product it modifies the interpretation and would definitely rule out any conclusion about the inhibitor specificity. In the preparation used to demonstrate the inhibitory properties of C8-substituted ATP analogs, in this work, we previously showed that a single catalytic site was involved in the sequential hydrolysis of the gamma and beta phosphate residues of ATP (35).

Reagents and solutions. ATP, tetramisole, malachite green, bovine serum albumin fraction V (BSA), CHAPS, sodium nitroprussiate (NaNP), (-) arterenol bitartrate (noradrenaline), heparin and indomethacin were obtained from Sigma Chemical Co. (St-Louis, MO, USA). ADP was obtained from Roche (Laval, QC., Canada), and Bradford reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). [Sar⁹, Met (O₂)¹¹] SP

77473-10

22

(NK-1) was synthesized by Dr. W. Neugebauer from the Université de Sherbrooke. Preparation of Krebs and phosphate buffer saline (PBS) were as followed. Krebs solution: 5.5 mM glucose, 117.5 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, pH 7.4. PBS: 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4 , pH 7.4. All the other reagents were of analytical grade and obtained from Sigma Chemical Co. (St-Louis, MO, USA).

10 **Isolation of particulate fractions.** Experiments were carried out with a particulate fraction obtained from bovine spleen according to the method of Sévigny et al. (37). Briefly, bovine spleens were cut in small pieces and homogenized (15-20%) with a Polytron in Tris-saline buffer supplemented with SBTI and
15 PMSF as protease inhibitors. After filtration with cheesecloth and centrifugation at 600 g the supernatant is centrifuged at 22,000 g for 90 min, and the resulting pellet is suspended in bicarbonate buffer and loaded on a sucrose cushion (40%) and centrifuged for two hours at 100,000 g. The fraction floating
20 on the cushion is harvested in five volumes of bicarbonate buffer. The pellet is suspended in Tris buffer/glycerol 7,5% and kept at $-20^\circ C$.

NTPDase assays. Enzyme activity was routinely
25 measured by the release of inorganic phosphorus with the malachite green colorimetric assay (61). Resistance to hydrolysis was measured at $37^\circ C$ in 1 ml of the following incubation medium: 8mM $CaCl_2$, 5 mM tetramisole, 50 mM Tris base, 50 mM imidazole, buffered at pH 7.6, and 100 μM of either ATP
30 or its analogs. Apparent K_m and V_{max} values for ATP, ADP and each of the hydrolysable purine nucleotide analogs were derived from Eadie and Hofstee plots, with substrate concentrations ranging between 10 and 300 μM for ATP and ADP, and between 15 and 100 μM for the analogs, unless stated otherwise. In both

77473-10

23

cases the reaction was started by the addition of 1.9 μ g of the enzyme preparation and stopped after 7 min with 250 μ L of the malachite green reagent. Apparent K_i values for non-hydrolysable purine nucleotide analogs were derived from Dixon replots, using inhibitor concentrations ranging from 0 to 100 μ M. Reactions were performed in the same incubation buffer, as previously described and were started by the addition of non-saturating ATP concentrations. Protein concentration was determined with the Bradford microplate assay using bovine serum albumin as a standard of reference (62).

To reduce potential artifacts resulting from the solubilisation of NTPDase by detergents, a particulate fraction (prepared as described above) was used. It is important to mention that there was no other detectable level of ATPase or ADPase activity in the preparation, other than that attributable to NTPDase. Also, possible alkaline phosphatase activity was inhibited by tetramisole added to the assays. In view of finding a specific and potent NTPDase inhibitor, two series of ATP analogs were examined, which were substituted at positions C2 and C8, respectively.

Analysis of NTPDase hydrolysis of ATP analogs. The ATP analogs substituted at positions C2 and C8, respectively, were first tested with respect to their resistance to NTPDase hydrolysis. The results obtained demonstrate that analogs substituted with electron donating groups at C8 were more resistant to hydrolysis than the corresponding C2 substituted analogs (Fig. 3). While a level of hydrolysis was observed with 8-Br-ATP, 8-BuNH-ATP, 7, 8-BuO-ATP, 8, and 8-ethylS-ATP, 6c (Fig. 7A), the compounds 8-cycloheptylS-ATP, 6a, 8-CH₂tBuS-ATP, 6b, 8-hexylS-ATP, 6d, and 8-BuS-ATP, 6e, were resistant to hydrolysis by NTPDase (Fig. 3). It is noteworthy that all the C2 substituted molecules displayed K_m values in the range found for ATP and ADP (Fig. 4). This indicates that the affinity for

77473-10

24

the catalytic site is equivalent for all these analogs. Hence the position of the substituent is clearly important for resistance to the catalytic activity of the enzyme.

Hydrophobic interactions and H-bonds of the C2 substituent appear to be important determinants for P2Y-R ligand affinity. The conformational preference of the ligands in solution, determined by NMR experiments, may explain in part the differences in P2Y-R potency between the 2- and 8-substituted compounds. All 2-substituted derivatives possess an anti conformation, whereas the 8-ether and thioether analogs are in the syn conformation. The latter are apparently not tolerated by the tentative P2Y1-R binding-site (64-66). In contrast, the NTPDase active site can accommodate 8-thioetherATP analogs, and even derivatives bearing large or bulky substituents (8-CH₂tBuS-ATP, and 8-cycloheptylS-ATP and 8-hexylS-ATP), probably in their expected syn conformation. This conformation is likely unfavorable for catalytic activity, namely, the orientation of the triphosphate chain in the syn conformation is probably shifted away from the catalytic amino acid residues.

Analysis of potency of C8-substituted ATP analogs as NTPDase inhibitors. Having established that these 8-thioether ATP derivatives were not significantly hydrolyzed, they were then investigated in regard to their potency as NTPDase inhibitors (Fig. 3). One striking feature is that the four derivatives, 6a, b, d, e are good inhibitors (Fig. 4-7). Again, the affinity (K_i) falls in the same range of affinity of that of ATP and ADP with one exception, the 8-CH₂tBuS-ATP, which has a slightly higher K_i (Fig. 4). However, even if these four analogs all display efficient NTPDase inhibition, 8-BuS-ATP, 6e, (Fig. 5) is to be considered as the preferred inhibitor, because it exhibits competitive inhibition with a K_i lower than those measured for compounds 6a, b, d, which are further less

77473-10

25

stable analogs with respect to NTPDase hydrolysis. The fact that this inhibitor (6e) interacts specifically with the binding site of the enzyme potentially reduces the risk of interference with other ATP-binding enzymes or receptors, and thus possesses a high degree of specificity. 8-CH₂tBuS-ATP, 6b, and 8-cycloheptylS-ATP, 6a, showed mixed types of inhibition thereby complicating the interpretation of their interaction with NTPDase (Fig. 6). Analog 6d exhibits non-competitive inhibition, thereby suggesting that its inhibitory effects are the results of an interaction with another part of the enzyme distinct from the catalytic site (Fig. 7B). From these results, it appears that the catalytic-site of NTPDase, may tolerate long and bulky substituents at the C8 position and also tolerate nucleotides in syn and anti conformations. It is noteworthy that the electronic nature of the modified purine ring has almost no influence on the affinity for the catalytic site, since thioether, aminoether and oxyether shows similar apparent affinity (K_m or K_i) for the enzyme.

Example 3: Analysis of effects on P2-receptor activity of purine nucleotide analogs.

Surgical Procedures. Dunkin-Hartley guinea pigs (300-350 g) of either sex were sacrificed by cervical dislocation according to the Canadian Council on Animal Care. The guinea pig mesentery was prepared as described by Berthiaume et al (63). Briefly, the colic and ileocolic branches of the superior mesenteric artery were tied and the superior mesenteric artery cannulated (Portex size tube 3FG). To isolate the mesenteric bed from the intestine, the mesentery was perfused (2 ml/min, for 5 min) via the mesenteric artery with a Krebs solution containing heparin (100 U/ml). The mesentery was then separated by cutting close to the intestine. A resting period of 60 min was then allowed during which the guinea pig mesenteric bed was

77473-10

26

perfused (2 ml/min) with a warmed (37°C) and gassed Krebs solution (95% O₂ and 5% CO₂) containing indomethacin (5 µM), as described earlier. In all the assays, perfusion pressure was increased to obtain a flow rate of 6 ml min⁻¹. Response of mesenteric bed, precontracted with noradrenaline (200 µM) in 0.9% saline solution, to the different drugs, was measured with a pressure transducer (Statham, model P-23AC) and recorded on a Grass physiograph (model 79D).

P2X-receptor assays. Guinea pig mesenteric bed was denuded from its endothelium layer by using 20 mM of CHAPS in PBS (63). Briefly, the CHAPS solution was infused for 45 sec, followed by a resting period of 30 min. Finally a second 45 sec infusion of 20 mM CHAPS was carried out. Blood vessels were then precontracted as earlier described. The efficiency of the endothelium removal technique was assessed by an intra-arterial bolus injection of 100 pmol of NK-1 in PBS. Reactivity of the media layer was confirmed by bolus injection of 3 nmol of NaNP (sodium nitroprussiate) in PBS. Bolus injections of increasing concentrations of 8-BuS-ATP (0.1 to 1000 pmol) in PBS, were administered. Variation of perfusion pressure were measured. Between each injection of 8-BuS-ATP a resting period was allowed to allow the return of pressure to baseline (i.e. precontracted pressure).

25

P2Y-receptor assays. Intact mesenteric bed vessels were precontracted with noradrenaline (200 µM). Mesentery was infused for 7 min with 7µM of 8-BuS-ATP or PBS (control), followed by intra-arterial bolus injection of increasing concentrations of ATP (0.1 pmol to 10000 pmol). A resting period between each ATP injection was allowed to return to baseline, as described above. Blood vessel reactivity was assessed by a bolus injection of 3 nmol of NaNP. Response of

77473-10

27

the endothelium layer was confirmed by injection of NK-1 (100 pmol).

Statistics. Data are expressed as mean \pm SEM and number of replicates are in figure legends. Kinetic studies have been performed using Graft software version 4 (Erithacus, UK). Unless stated otherwise, comparison between data was performed by one-way ANOVA test. Probability values of less than 0.05 were considered significant.

10

Effects on purinoceptor activity. Using the methods described above, the compounds of the invention were analyzed with respect to any effects on the activity of purinoceptors. Recent studies have indicated that 8-BuS-ATP was a poor agonist for P2Y₁ receptor. In rat astrocytes this compound has no effect on $[Ca^{2+}]_i$ level, whereas the corresponding 2-substituted ATP analogs potently increased $[Ca^{2+}]_i$ level. Similar results were obtained with turkey erythrocyte membranes (67). Since this molecule qualifies as a potent inhibitor, we tested its influence in the isolated mesenteric bed of the guinea pig for potential P2X and P2Y purinergic effects was tested (Fig. 8). Isolated guinea pig mesenteric artery and vein have been shown to respond to ATP via a P2X-purinoceptor located at the surface of the smooth muscle cells (68, 69). This latter purinoceptor was sensitive to α , β -CH₂-ATP, 1b, a P2X agonist (70, 71). CHAPS was used to remove the blood vessel endothelium which gives rise to endothelium denuded vessels. Non-functional endothelium has been shown by a lack of response to 100 pmol of NK-1 (63). Our data show that 8-BuS-ATP was not able to initiate any P2X-R effect at concentrations up to 1000 pmol, indicating that it does not interact with P2X-R (Fig. 8A). We also evaluated the effect of an infusion of 8-BuS-ATP on the vasodilatory response induced by administration of increasing ATP concentrations (0.1 pmol to 10000 pmol), in intact

006090" 2476560

77473-10

28

mesenteric bed. Even in the presence of 7 μ M 8-BuS-ATP the response to ATP was unmodified, confirming that 8-BuS-ATP did not interact with P2Y-R (Fig. 8B).

The integrity of the endothelium was assessed and
5 confirmed with NK-1 and NanP (Fig. 8C).

All of the references cited above and listed in the REFERENCES section below are herein incorporated by reference.

005050" 427690

77473-10

29

REFERENCES

1. Burnstock, G., Campbell, G., Bennett, M., and Holman, M.E. *Int. J. Neuropharmacol* 3: 163-166, 1964.
- 5 2. Burnstock, G. Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. *Pharmacol. Rev.* 21 (4): 247-324, 1969.
3. Su, C., Bevan, J.A., and Burnstock, G. [3H] adenosine triphosphate: release during stimulation of enteric
10 nerves. *Sciences* 173(994): 336-338, 1971.
4. Langer, S.Z., and Pinto, J.E.B. Possible involvement of a transmitter different from norepinephrine in the residual responses to nerves stimulation of the cat nictitating membrane after pretreatment with reserpine. *J. Pharmacol. Exp. Ther.* 196(3): 697-713, 1976.
15
5. Burnstock, G. Purinergic receptors. *J. Theor. Biol.* 62 (2): 491-503, 1976.
6. Von Kugelgen, I., and Starke, K. Noradrenaline-ATP co-transmission in the sympathetic nervous system. *Trends Pharmacol. Sci.* 12(9): 319-324, 1991.
20
7. Westfall, D.P., Sedaa, K.O., Shinozuka, K., Bjun, R.A., and Buxton, I.L. *Ann. NY Acad. Sci.* 603: 300-310, 1990.
8. Burnstock, G. Neural nomenclature. *Nature* 229(5282): 282-283, 1971.
25
9. Burnstock, G. Purinergic receptors. *J. Theor. Biol.* 62 (2): 491-503, 1976.
10. Burnstock, G. A basis for distinguishing two types of purinergic receptors. In: *Cell membrane receptors for drugs and hormones: A multidisciplinary approach.* (Eds. R.W. Straub and L. Bolis), Raven press, New York. Pp. 108-118, 1978.
30
11. Fredholm, B.B., Abbracchio, M.P., Burnstosk, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., and

0059117 060900

77473-10

30

- Williams, M. Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* 46(2): 143-156, 1994.
12. Juul, B., Plesner, L., and Aalkjaer, C. Effects of ATP and related nucleotides on the tone of isolated rat mesenteric resistance arteries. *J. Pharmacol. Exp. Therap.* 264: 1234-1240, 1993.
13. Motte, S.; Commun, D.; Pirotton, S.; Boeynaems, J.M. Involvement of multiple receptors in the actions of extracellular ATP: the example of vascular endothelial cells. *Int. J. Biochem. Cell Biol.* 27: 1-7, 1995.
14. Rongen, G.A., Floras, J.S., Lender, J.W.M., Thier, T., and Smits, P. Cardiovascular pharmacology of purines. *Clin. Sci.* 92: 13-24, 1997.
15. Dubyak, G.R., and El Moatassim, C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* 265: C577-C606, 1993.
16. Johnson, C.R., and Hourani, S.M. Contractile effects of uridine 5'-triphosphate in the rat duodenum. *Br. J. Pharmacol.* 113(4): 1191-1196, 1994.
17. Pennanen, M.F., Bass, B.L., Dziki, A.J., and Harmon, J.W. Adenosine differential effect on blood flow to suregions of the upper gastrointestinal tract. *J. Surg. Res.* 56(5): 461-465, 1994.
- 25 18. Strohmeier, G.R., Reppert, S.M., Lencer, W.I., and Madana, J.L. The A_{2b} adenosine receptor mediated cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J. Biol. Chem.* 270(5): 2387-2394, 1995.
19. Hancock, D.L., and Coupar, I.M. Functional characterization of the adenosine receptor mediating inhibition of peristalsis in the rat jejunum. *Br. J. Pharmacol* 115(5):739-744, 1995.
- 30 20. Sarna, S.K. Gastrointestinal longitudinal muscle contractions. *Am. J. Physiol.* 265(1pt1): G156-G164, 1993.

77473-10

31

21. Baricordi, O.R., Ferrari, D., Melchiorri, L.,
Chiozzi, P., Hamann, S., Chial, E., Rubini, M., and Di
Virgilio, F. An ATP-activated channel is involved in
mitogenic stimulation of human T lymphocytes. *Blood* 87(2):
5 682-690, 1996.
22. Di Virgilio, F. The P2Z purinoceptor: an intriguing
role in immunity, inflammation and cell death. *Immunol.*
Today 16(11): 525-528, 1995.
23. Ventura, M.A., and Thomopoulos, P. ADP and ATP
10 activate distinct signaling pathways in human promonocytic U-
937 cells differentiated with 1,25-dihydroxy-vitamin D3.
Mol. Pharmacol 47: 104-114, 1995.
24. Biffen, M., and Alexander, D.R. Mobilization of
intracellular Ca²⁺ by adenine nucleotides in human T-
15 leukaemia cells: evidence for ADP-specific and P2y-purinergic
receptors. *Biochem. J.* 304:769-774, 1994.
25. Apasov, S., Koshiba, M., Redegeld, F., and
Sitokovsky, M.V. Role of extracellular ATP and P1 and P2
classes of purinergic receptors in T-cell development and
20 cytotoxic T lymphocyte effector functions. *Immunol. Rev.*
146: 5-19, 1995.
26. Hedge, S.S., Mandel, D.A., Wilfird, M.R., Briand, S.,
Ford, A.P.D.W., and Eglen, R.M. Evidence for purinergic
neutransmission in the urinary bladder of pithed rats. *Eur.*
25 *J. Pharmacol.* 349(1): 75-82, 1998.
27. Dunwiddie, T.V., Abbracchio, M.P., Bischofberger, N.,
Brundage, J.M., Bruell, G., Collo, G., Corsi, C., Diao, L.,
Kawashima, E., Jacobson, K.A., Latini, S., Lin, R.C.S., Noth,
R.A., Pazzagli, M., Pedata, F., Pepen, G.C., Proctor, W.R.,
30 Rassendren, F., Surprenant, A., and Cattabeni, F.
Purinoceptors in the central nervous system. *Drug Dev. Res.*
39(3-4): 361-370, 1996.
28. Burnstock, G., and Wood, J.N. Purinergic receptors-
Their role in nociception and primary afferent

0059177-060900

77473-10

32

- neurotransmission. *Curr. Opinion in Neurobiol.* 6(4): 526-532, 1996.
29. Von Kugelgen, I. Purinoceptors modulating the release of noradrenaline. *J. Autonomic. Pharmacol.* 14(1): 11-12, 1994.
30. Beaudoin, A.R.; Sévigny, J.; Picher, M. ATP diphosphohydrolases, apyrases and nucleotide phosphohydrolases: biochemical properties and functions. In: *Biomembrane*, vol. 5; Lee, A.G., Ed.; Greenwich, CT: JAI, pp. 369-401, 1996.
31. Beaudoin, A.R.; Grondin, G.; Enjyoji, K.; Robson, S.C.; Sévigny, J.; Fischer, B.; Gendron, F.-P. Physiological role of NTPDases (ATP diphosphohydrolases) in mammals. *Proceeding of the 2nd International Workshop on ecto-ATPase and related nucleotidases*. Diepenbeek, Belgium, 14-18 June 1999. Vanduffel L., and Lemmens R., Eds. Shaker Publishing B.V., The Netherlands; pp. 125-135, 2000.
32. Plesner, L. Ecto-ATPases: identities and functions. *Int. Rev. Cytol.* 158: 141-214, 1995.
33. Vlakovic, S.M.; Thorne, P.R.; Hously, G.D.; Munoz, D.J.B.; Kendrick, I.S. Ecto-nucleotidases terminate purinergic signalling in the cochlear endolymphatic compartment. *Neuroreport* 9: 1559-1565, 1998.
34. Zimmermann, H. 5'-Nucleotidase: molecular structure and functional aspects. *Biochem. J.* 285: 345-365, 1992.
35. Laliberté, J.F., and Beaudoin AR. Sequential hydrolysis of the gamma- and beta-phosphate groups of ATP by the ATP diphosphohydrolase from pig pancreas. *Biochim. Biophys Acta.* 742(1):9-15, 1983.
36. Côté Y.P., Pavate C. and Beaudoin A.R. The control of nucleotides in blood vessels: Role of the ATP diphosphohydrolase (apyrase). *Curr. Top. Pharmacol.* 1: 83-92, 1992.

77473-10

33

37. Sévigny J., Levesque F.R., Grondin G. and Beaudoin A.R. Purification of the blood vessel ATP diphosphohydrolase, identification and localization by immunological techniques. *Biochim. Biophys. Acta* 1334: 73-88, 1997.
38. LeBel D., Poirier G.G. Phaneuf, S. St-Jean P., Laliberté J.-F. and Beaudoin A.R. Characterization and purification of a calcium sensitive ATP diphosphohydrolase from the pig pancreas. *J. Biol. Chem.* 255: 1227-1233, 1980.
39. Sévigny, J.; Côté, Y.P.; Beaudoin, A.R. Purification of pancreas type I ATP diphosphohydrolase and identification by affinity labelling with 5'-p-fluorosulfonyl benzoyl adenosine ATP analog. *Biochem. J.* 312: 351-356, 1997.
40. Sévigny J., Levesque F.R., Grondin G. and Beaudoin A.R. Purification of the blood vessel ATP diphosphohydrolase, identification and localization by immunological techniques. *Biochim. Biophys. Acta* 1334: 73-88, 1997.
41. Christoforidis, S.; Papamarcaki, T.; Galaris, D.; Kellner, R.; Tsolas, O. Purification and properties of human placental ATP diphosphohydrolase. *Eur. J. Biochem.* 234: 66-74, 1995.
42. Kaczmarek, E.; Koziack, K.; Sévigny, J.; Siegel, J.B.; Anrather, J.; Beaudoin, A.R.; Bach, F.H.; Robson, S.C. Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J. Biol. Chem.* 271: 33116-33122, 1996.
43. Maliszewski, C.R.; Delespesse, G.L.; Schoenborn, M.A.; Armitage, R.J.; Fanslow, W.C.; Nakajima, T.; Baker, E.; Sutherland, G.R.; Poindexter, K.; Birks, C.; Alpert, A.; Friend, D.; Gimpel, S.D.; Gayle III, R.B. The CD39 lymphoid cell activation antigen: Molecular cloning and structural characterization. *J. Immunol.* 153: 3574-3583, 1994.
44. Wang, T.F.; Guidotti, G. CD39 is an ecto-(Ca²⁺, Mg²⁺)-ATPase. *J. Biol. Chem.* 271: 9898-9901, 1996.

77473-10

34

45. Barcellos, C.K. Schetinger MR. Battastini AM. Silva LB. Dias RD. Sarkis JJ. Inhibitory effect of cadmium acetate on synaptosomal ATP diphosphohydrolase (EC 3.6.1.5; apyrase) from adult rat cerebral cortex. Br. J. Med and Biol. Res. 5 27(5): 1111-1115, 1994.
46. Côté, Y.P., Ouellet, S., and Beaudoin, A.R. Kinetic properties of type-II ATP diphosphohydrolase from the tunica media of the bovine aorta. Biochim. Biophys. Acta 1160(3): 246-250, 1992.
- 10 47. Picher, M.; Sévigny, J.; D'Orléans-Juste, P.; Beaudoin, A.R. Hydrolysis of P2-purinoceptor agonists by a purified ectonucleotidase from the bovine aorta, the ATP diphosphohydrolase. Biochem. Pharmacol. 51: 1453-1460, 1996.
- 15 48. Westfall, T.D., Kennedy, C., and Sneddon, P. The ecto-ATPase inhibitors ARL 67156 enhance parasympathetic neurotransmission in the guinea-pig urinary bladder. Eur. J. Pharmacol. 329(2-3): 169-173, 1997.
- 20 49. Crack, B.E., Pollard, C.E., Beukers, M.W., Roberts, S.M., Hunt, S.F., Ingall, A.H., McKechnie, K.C., Ijzerman, A.P., and Leff, P. Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. Br. J. Pharmacol. 114(2): 475-481, 1995.
- 25 50. Chen, B.C., Lee, C.M., and Lin W.W. Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C-6 glioma cells and raw 264.7 macrophages. Br. J. Pharmacol. 119(8): 1628-1634, 1996.
51. Kennedy, C., Westfall, T.D., and Sneddon, P. Modulation of purinergic neurotransmission by ecto-ATPase. Sem. Neurosci. 8(4): 195-199, 1996.
- 30 52. Fischer, B.; Chulkin, A.; Boyer, J.L.; Harden, K.T.; Gendron, F.-P.; Beaudoin, A.R.; Chapal, J.; Hillaire-Buys, D.; Petit, P. 2-thioether-5'-O-(1-thiotriphosphate) adenine derivatives as new insulin secretagogues acting through P2Y-receptors. J. Med. Chem. 42: 3636-3646, 1999.

77473-10

35

53. Bültmann, R., Wittenburg, H., Pause, B., Kurz, G., Nickel, P., and Starke, K. P2-purinoceptors antagonists: III. Blockade of P2-purinoceptor subtypes and ecto-nucleotidases by compounds related to suramin. Naunyn-Schmiedeberg's Arch. Pharmacol. 354: 498-504, 1996.
54. Tuluc, F., Bültmann, R., Glänzel, M., Wilhelm Frahm, A., and Starke, K. P2-receptor antagonists: IV. Blockade of P2 receptor subtypes and ecto-nucleotidases by compounds related to reactive blue 2. Naunyn-Schmiedeberg's Arch. Pharmacol. 357: 111-120, 1998.
55. Bültmann, R., and Starke, K. Reactive red 2: a P2Y-selective purinoceptor antagonist and an inhibitor of ecto-nucleotidase. Naunyn-Schmiedeberg's Arch. Pharmacol. 352: 477-482, 1995.
56. Wittenburg, H., Bültmann, R., Pause, B., Ganter, C., Kurz, G., and Starke, K. P2-purinoceptor antagonists: II. Blockade of P2-purinoceptor subtypes and ecto-nucleotidases by compounds related to Evans Blue and trypan blue. Naunyn-Schmiedeberg's Arch. Pharmacol. 354: 491-497, 1996.
57. Bültmann, R., Pause, B., Wittenburg, H., Kurz, G., and Starke, K. P2-purinoceptor antagonists: I. Blockade of P2-purinoceptor subtypes and ecto-nucleotidases by small aromatic isothiocyanato-sulphonates. Naunyn-Schmiedeberg's Arch. Pharmacol. 354: 481-490, 1996.
58. Bonan, C.D., Battastini, A.M.O., Schetinger, M.R.C., Moreira, C.M., Frassetto, S.S., Dias, R.D., and Sarkis, J.J.F. Effects of 9-amino-1,2,3,4-tetrahydroacridine (THA) on ATP diphosphohdrolase (EC 3.6.1.5) and 5' nucleotidases (EC 3.1.3.5) from rat brain synaptosomes. Gen. Pharmac. 28(5): 761-766, 1997.
59. Gendron F.-P., Halbfinger E., Fischer B., D'Orleans-Juste P., Duval M. and Beaudoin, A. R. Novel ATP diphosphhydrolase inhibitors: Synthesis, biochemical and

77473-10

36

pharmacological characterization. J. Med. Chem., 2000. (in press).

60. Fischer, B.; Boyer, J.L.; Hoyle, C.H.; Ziganshin, A.U.; Brizzolara, A.L.; Knight, G.E.; Zimmet, J.; Burnstock, G.; Harden, T.K.; Jacobson, K.A. Identification of potent, selective P2Y-purinoceptor agonists: structure-activity relationships for 2-thioether derivatives of adenosine 5'-triphosphate. J. Med. Chem. 36: 3937-3946, 1993.
61. Baykov A.A., Evtushenko O.A. and Avaeva S.M. Malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal. Biochem. 171: 266-270, 1988.
62. Bradford M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.
63. Berthiaume, N.; Claing, A.; Regoli, D.; Warner, T.D.; D'Orléans-Juste, P. Characterization of receptors for kinins and neurokinins in the arterial and venous mesenteric vasculatures of the guinea-pig. Br. J. Pharmacol. 115: 1319-1325, 1995.
64. Halbfinger, E.; Major, D.T.; Ritzmann, M.; Uhl, J.; Reiser, G.; Boyer, J.L.; Harden, K.T.; Fischer, B. Molecular recognition of modified adenine nucleotides by the P2Y₁-receptor. Part I. A synthetic, biochemical, and NMR approach. J. Med. Chem. 42: 5325-5337, 1999.
65. Major, D.T.; Halbfinger, E.; Fischer, B. Molecular recognition of modified adenine nucleotides by the P2Y₁-receptor. II. A computational approach J. Med. Chem. 42: 5338-5347, 1999.
66. Van Rhee, A.M.; Fischer, B.; Van Galen, P.J.M.; Jacobson, K.A. Modelling the P2Y purinoceptor using rhodopsin as template. Drug Design and Delivery 13: 133-154, 1995.

77473-10

37

67. Fischer, B.; Chulkin, A.; Boyer, J.L.; Harden, K.T.; Gendron, F.-P.; Beaudoin, A.R.; Chapal, J.; Hillaire-Buys, D.; Petit, P. 2-thioether-5'-O-(1-thiotriphosphate) adenine derivatives as new insulin secretagogues acting through P2Y-
5 receptors. J. Med. Chem. 42: 3636-3646, 1999.
68. Hirst, G.D.S.; Jobling, P. The distribution of γ -adrenoceptors and P2 purinoceptors in mesenteric arteries and
vains of the guinea-pig. Br. J. Pharmacol. 96: 993-999, 1989.
69. Onaka, U.; Fujii, K.; Abe, I.; Fujishima, M.
10 Enhancement by exogenous and locally generated angiotensin II
of purinergic neurotransmission via angiotensin type 1
receptor in the guinea-pig isolated mesenteric artery. Br.
J. Pharmacol. 122: 942-948, 1997.
70. Fujii, K. Evidence for adenosine triphosphate as an
15 excitatory transmitter in guinea-pig, rabbit and pig urinary
bladder. J. Physiol. 404: 39-52, 1989.
71. Ishikawa, S. Actions of ATP and α , β -methylene ATP
on neuromuscular transmission and smooth muscle membrane of
the rabbit and guinea-pig mesenteric arteries. Br. J.
20 Pharmacol. 86: 777-787, 1985.

005090 276560

77473-10

38

CLAIMS:

1. A C8-substituted purine nucleotide analog, wherein
the analog is substituted at the C8 position with a substituent
5 other than H.
2. The analog of claim 1, wherein the purine is adenine.
3. The analog of claim 1, wherein the substituent is an
10 ether, thioether or an amine.
4. The analog of claim 2, wherein the substituent is an
ether, thioether or an amine.
- 15 5. The analog of claim 1, wherein the substituent is an
ether, and wherein the ether substituent has the structure:
-O-X.
6. The analog of claim 5, wherein X is an alkyl group.
20
7. The analog of claim 6, wherein X is selected from the
group consisting of:
- (a) C_7H_{13} (cycloheptyl)
- (b) $(CH_3)_3CCH_2$
- 25 (c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$

77473-10

39

8. The analog of claim 1, wherein the substituent is a thioether, and wherein the thioether substituent has the structure:

-S-X.

5

9. The analog of claim 8, wherein X is an alkyl group.

10. The analog of claim 9, wherein X is selected from the group consisting of:

- 10 (a) C_7H_{13} (cycloheptyl)
(b) $(CH_3)_3CCH_2$
(c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$

11. The analog of claim 1, wherein the substituent is an amine, and wherein the amine substituent has the structure:

-NH-X.

12. The analog of claim 11, wherein X is an alkyl group.

13. The analog of claim 12, wherein X is selected from the group consisting of:

- (a) C_7H_{13} (cycloheptyl)
(b) $(CH_3)_3CCH_2$
(c) $CH_3(CH_2)_n$, wherein $1 \leq n \leq 5$

25

77473-10

40

14. The analog of claim 1 selected from the group consisting of:

compound 6a, compound 6b, compound 6c, compound 6d,
compound 6e, compound 7a, compound 7b, compound 7c,
5 compound 7d, compound 7e, compound 8a, compound 8b,
compound 8c, compound 8d, and compound 8e.

15. A method for modulating the activity of an NTPDase enzyme comprising exposing the enzyme to the analog according
10 to claim 1.

16. The method according to claim 15 wherein the activity of the NTPDase enzyme is inhibited.

15 17. A method for modulating the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in a biological system, comprising the step of introducing into said system the analog according to claim 1.

20

18. A method for modulating the activity of a biological process in a biological system, wherein said process is affected by the level of purine nucleotide(s) and/or nucleoside(s) and/or metabolite(s) or derivative(s) thereof in
25 said system, comprising the step of introducing into said system the analog according to claim 1.

77473-10

41

19. The method of claim 18, wherein the biological process is aggregation and thrombogenicity.

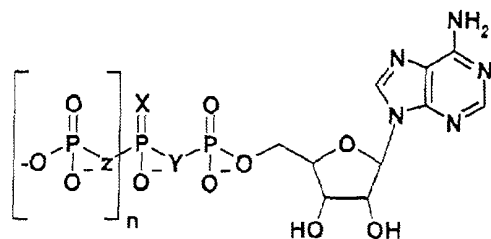
005090" 476360

ABSTRACT

Ectonucleoside triphosphate diphosphohydrolases [NTPDases; EC 3.6.1.5] constitute a family of enzymes which are involved in the metabolism of extracellular nucleotides, catalysing the hydrolysis of the gamma and beta phosphate bonds of triphospho- and diphosphonucleosides (whereas 5'nucleotidases [EC 3.1.3.5] catalyse the hydrolysis of alpha phosphate bond of monophosphonucleosides). These extracellular nucleotides interact with endothelial, epithelial and smooth muscle cells, as well as blood cells and lymphoid cells, to influence the different physiological systems of vertebrates. Since these ecto-nucleotidases alter the extracellular concentrations of nucleotides these enzymes modulate their physiological effects, including, for example, platelet aggregation, heart function, control of vascular tone and inflammation reactions, electrolyte secretion and gastrointestinal motility, neurotransmission both in central and peripheral nervous systems, as well as other effects in other physiological systems. This invention provides C8 substituted purine nucleotide analogues, such as ATP analogues, and further provides their use as inhibitors of NTPDases and thereby as tools to modulate the conversion of nucleotides into nucleoside derivatives, and thus modulate the levels of these compounds. Such modulation further provides for the modulation of the activity and function of many processes which are affected by these compounds.

00591177-060900

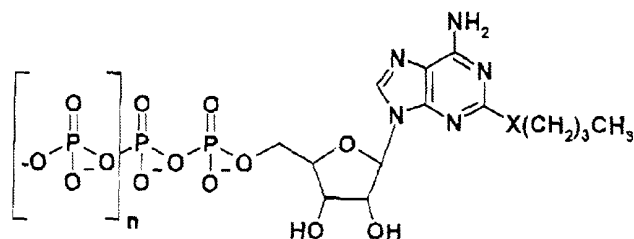
1/8



1 a : X = S, Y = O, Z = O, n = 0

b : X = O, Y = CH₂, Z = O, n = 1c : X = O, Y = O, Z = CH₂, n = 1d : X = S, Y = CH₂, Z = NH, n = 1

Fig. 1A



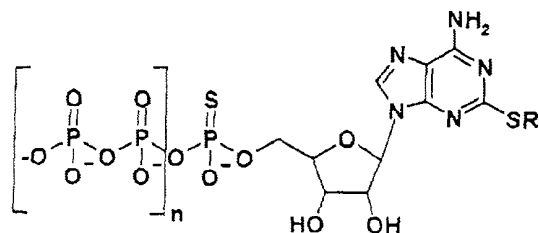
2 a : n = 1, X = S

b : n = 0, X = S

c : n = 1, X = NH

d : n = 1, X = O

Fig. 1B



3. n = 1, a : R = hexyl, b : R = benzyl

4. n = 0, a : R = hexyl, b : R = benzyl

Fig. 1C

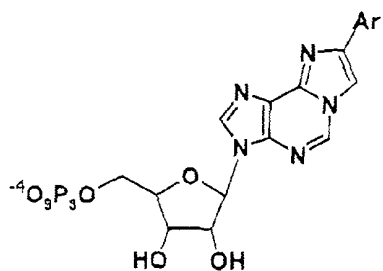
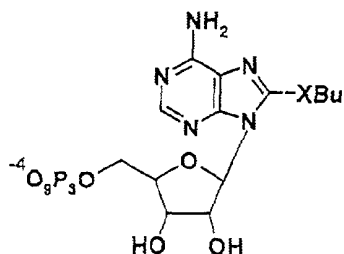
5 a : Ar = p-NO₂-C₆H₄b : Ar = p-NH₂-C₆H₄

Fig. 1D



6. X = S

7. X = NH

8. X = O

Fig. 1E

2/8

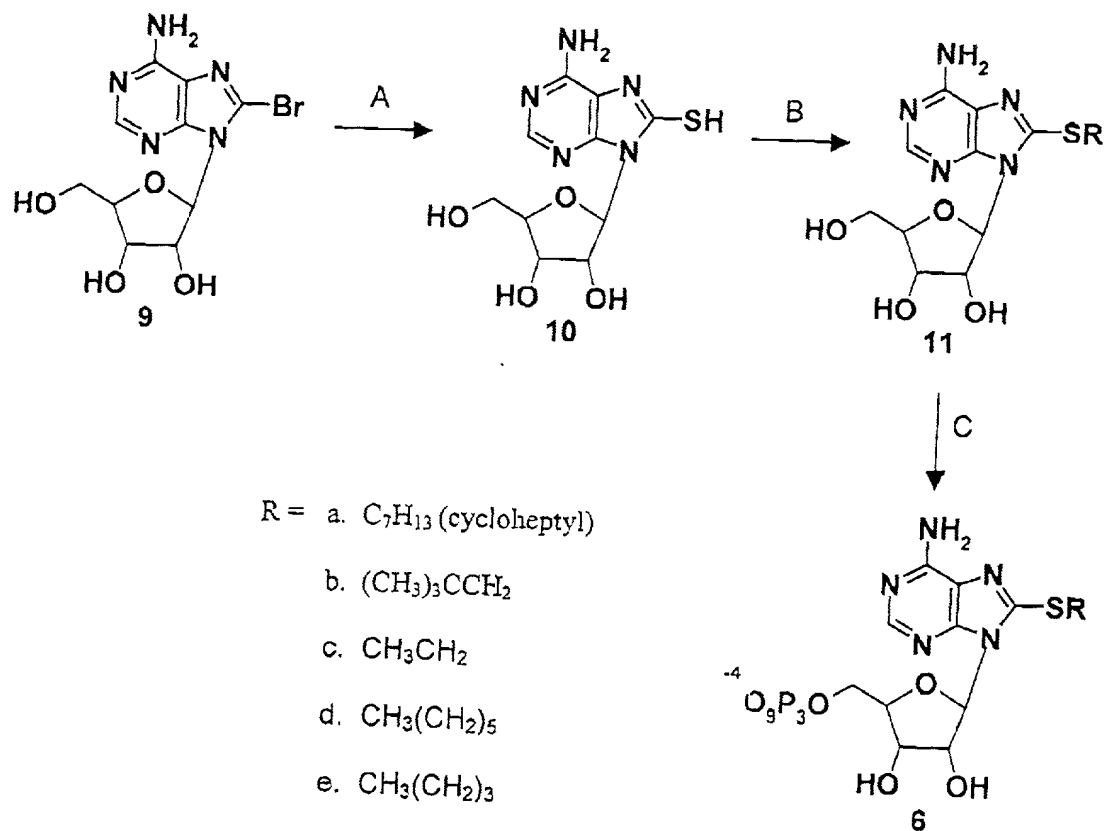


Fig. 2

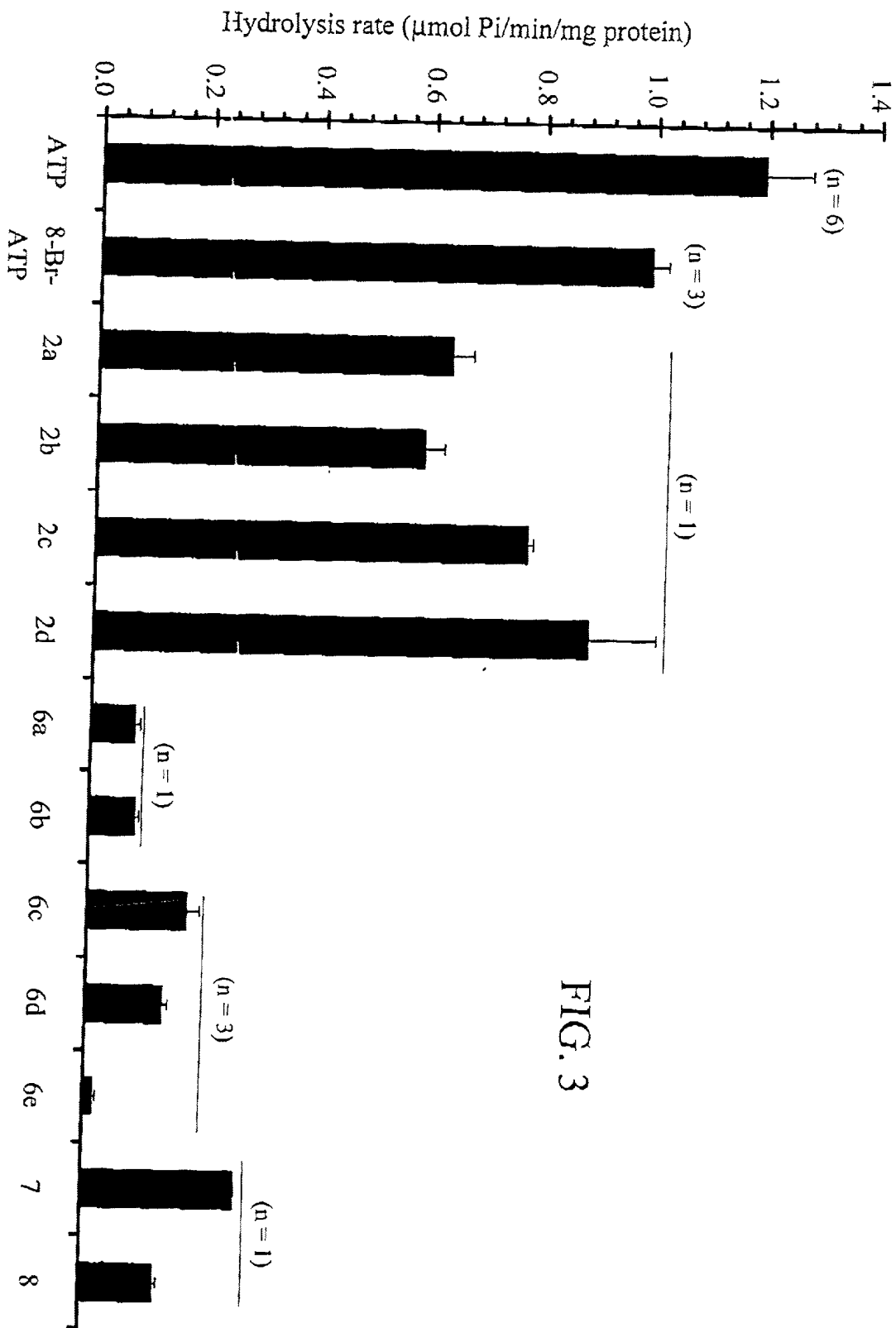


FIG. 3

4/8

Substrates	K _m (μ M)	V _{max} (μ mol/min/mg protein)	Inhibitors	K _i (μ M)
ATP	18 \pm 1	1.65 \pm 0.10	8-cycloheptylS-ATP	31 \pm 2.5
ADP	33 \pm 1	1.30 \pm 0.08	8-CH ₂ BuS-ATP	45 \pm 2.5
2-BuS-ATP	36 \pm 6	0.83 \pm 0.05	8-hexylS-ATP	16 \pm 2.0
2-BuS-ADP	63 \pm 14	0.94 \pm 0.10	8-BuS-ATP	10 \pm 2.0
2-BuNH-ATP	32 \pm 8	0.99 \pm 0.10		
2-BuO-ATP2d	28 \pm 8	0.82 \pm 0.09		
8-bromo-ATP	22 \pm 5	0.63 \pm 0.04		
8-ethylS-ATP	12 \pm 5	0.30 \pm 0.03		
8-BuNH-ATP	20 \pm 7	0.28 \pm 0.03		
8-BuO-ATP	26 \pm 5	0.20 \pm 0.01		

FIG. 4

5/8

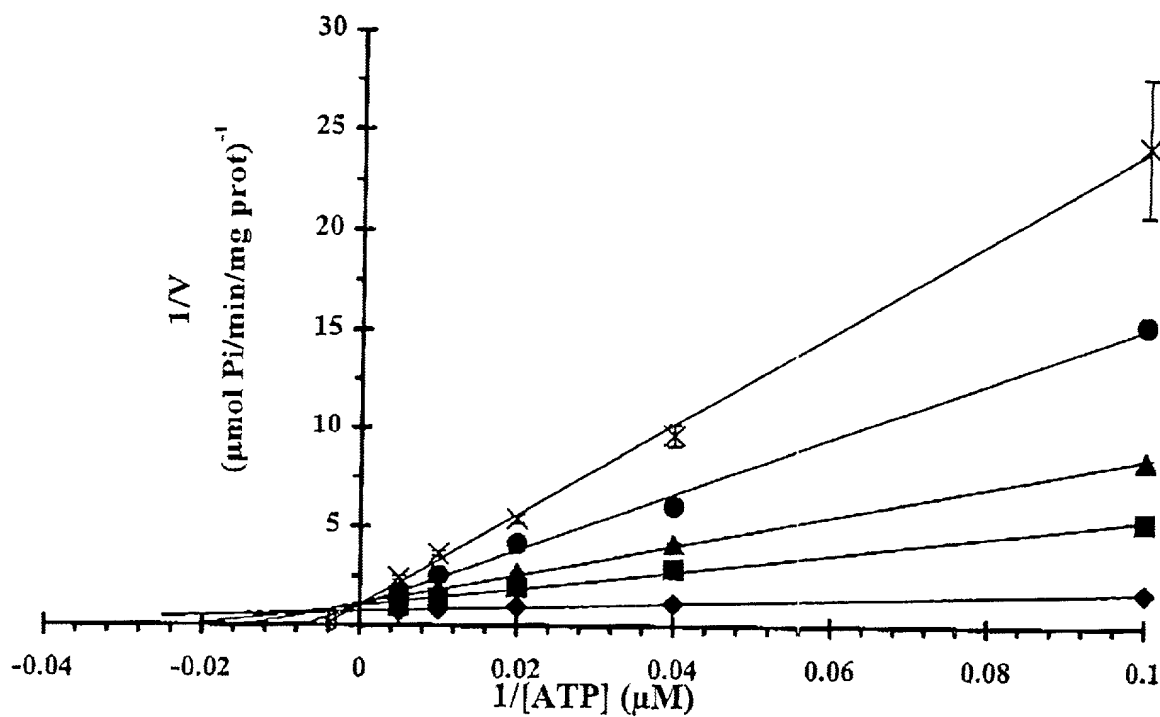


FIG. 5A

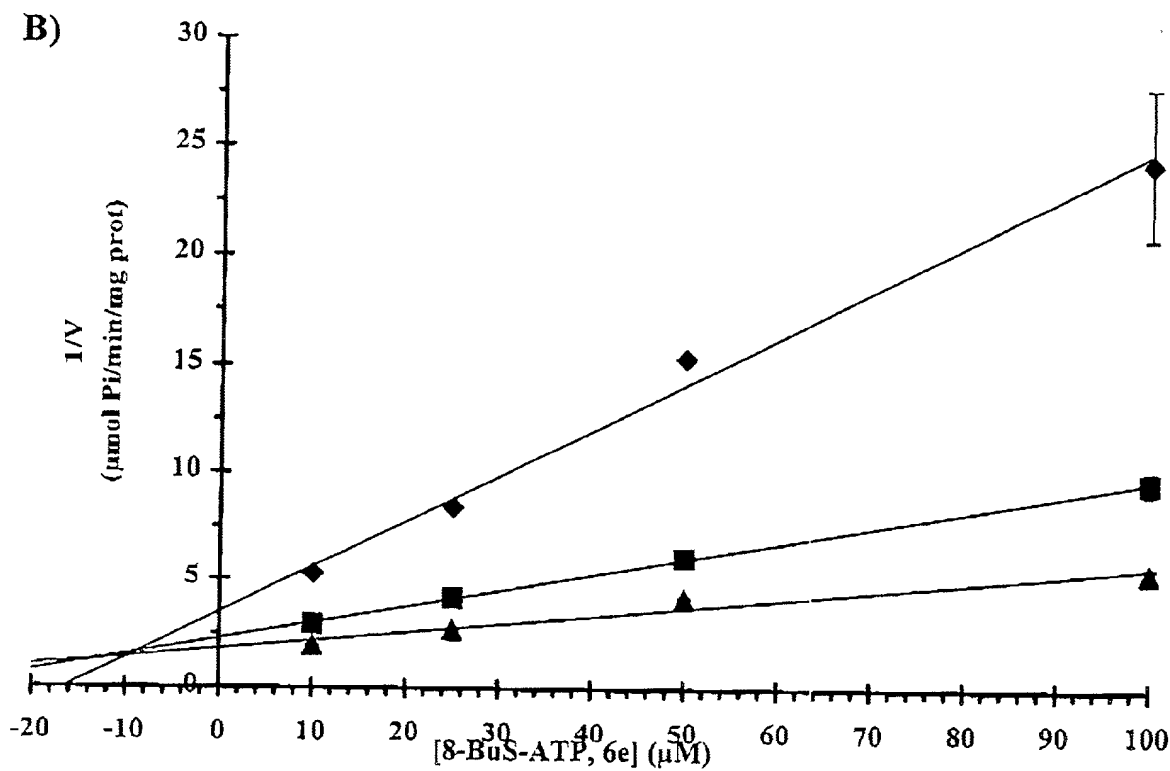


FIG. 5B

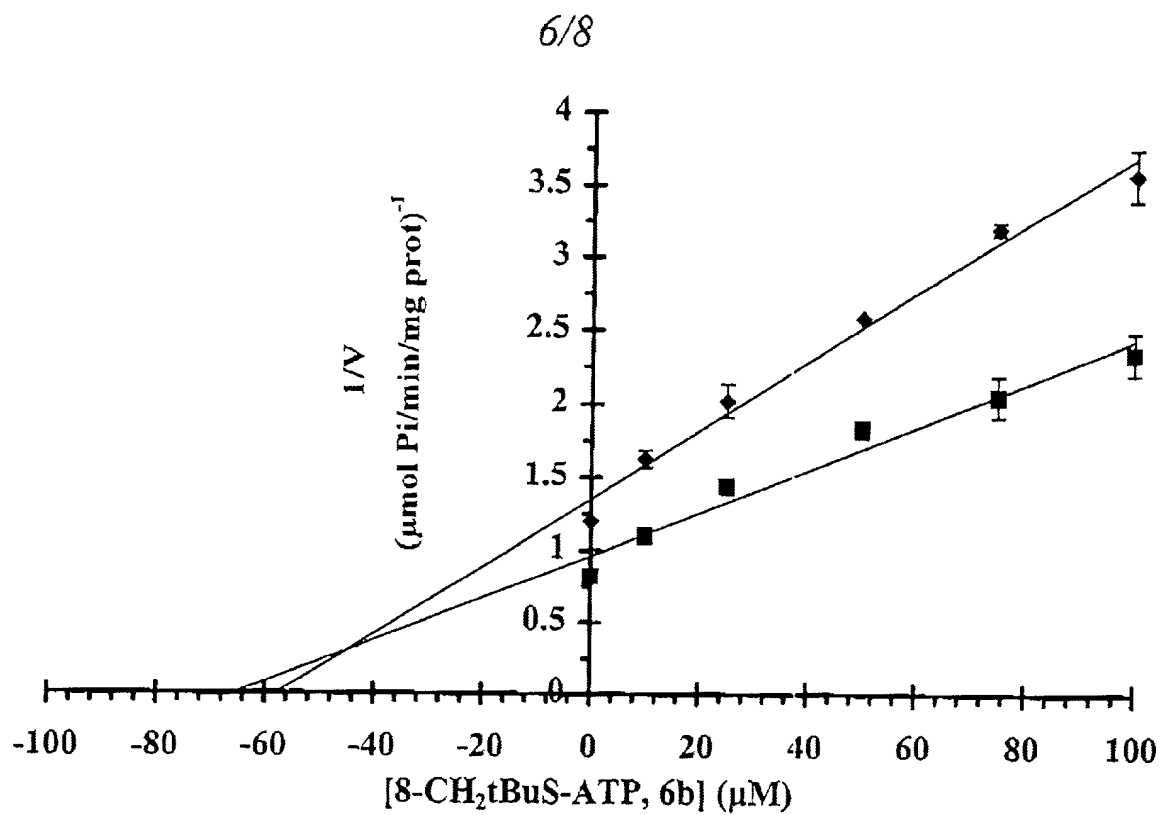


FIG. 6A

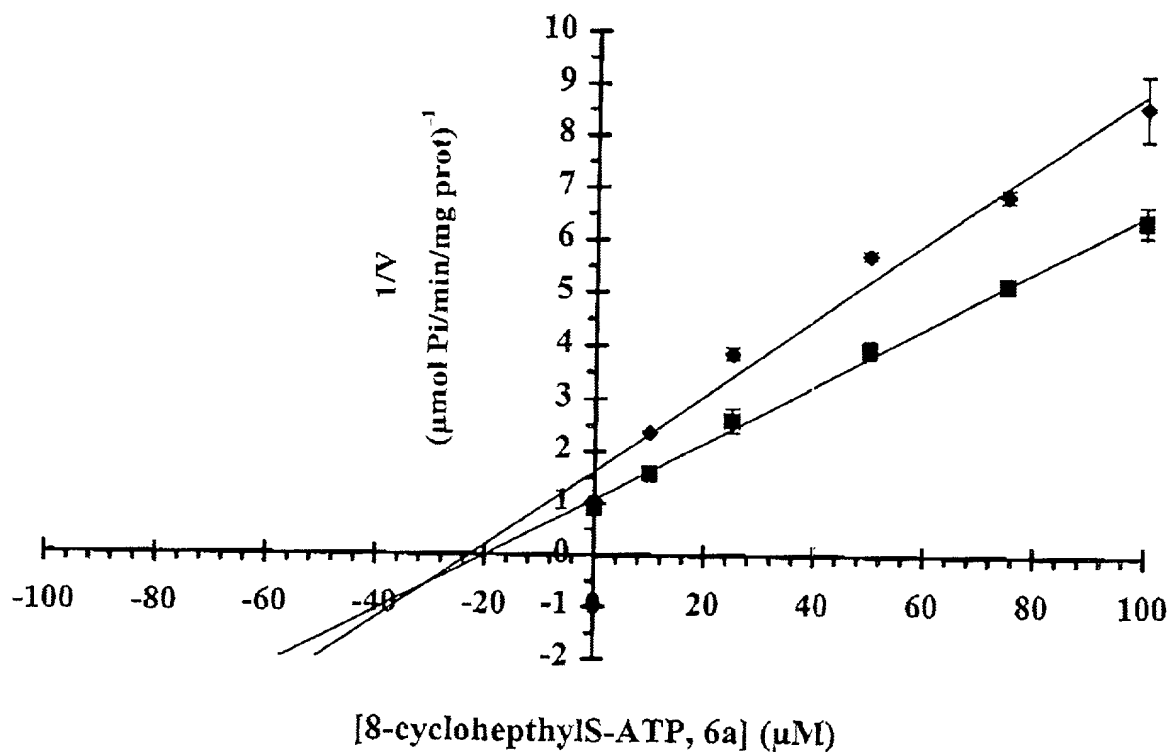


FIG. 6B

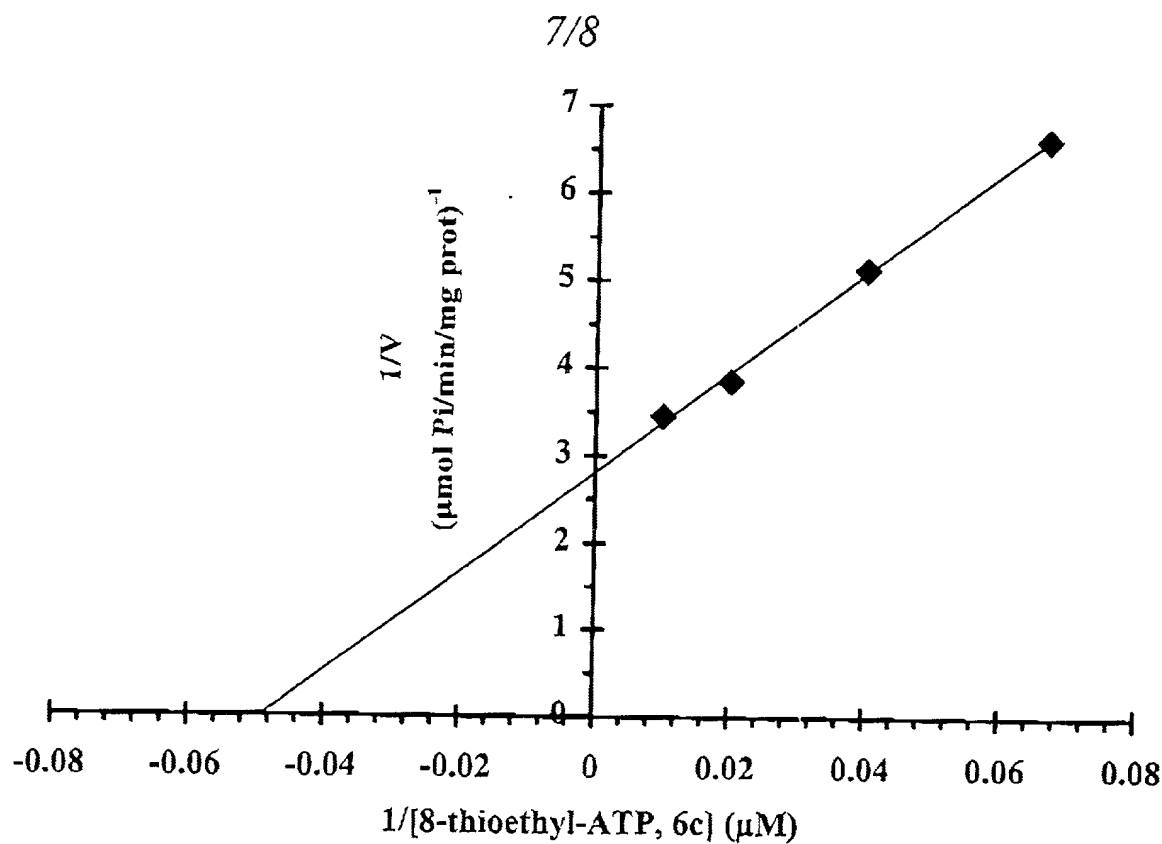


FIG. 7A

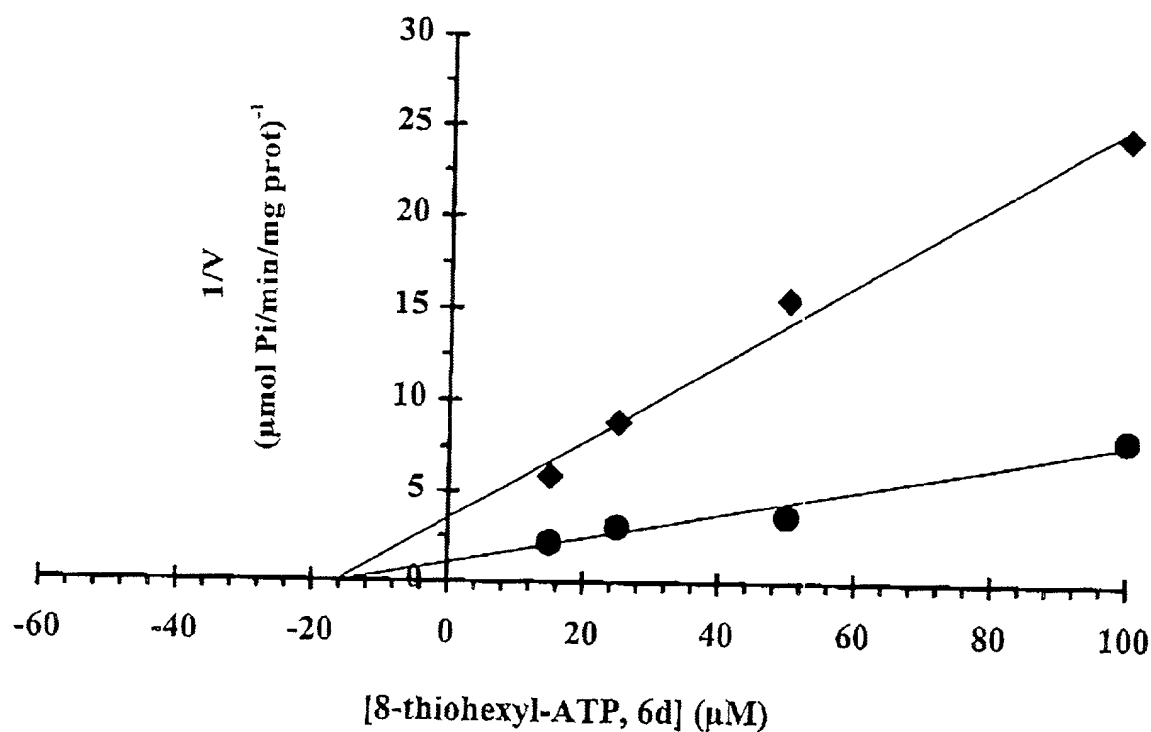


FIG. 7B

8/8

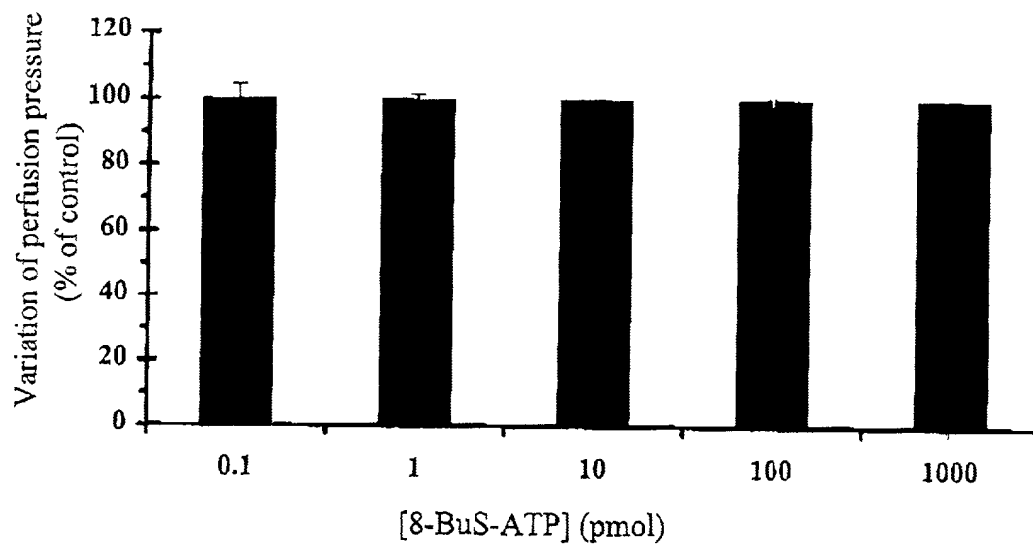


FIG. 8A

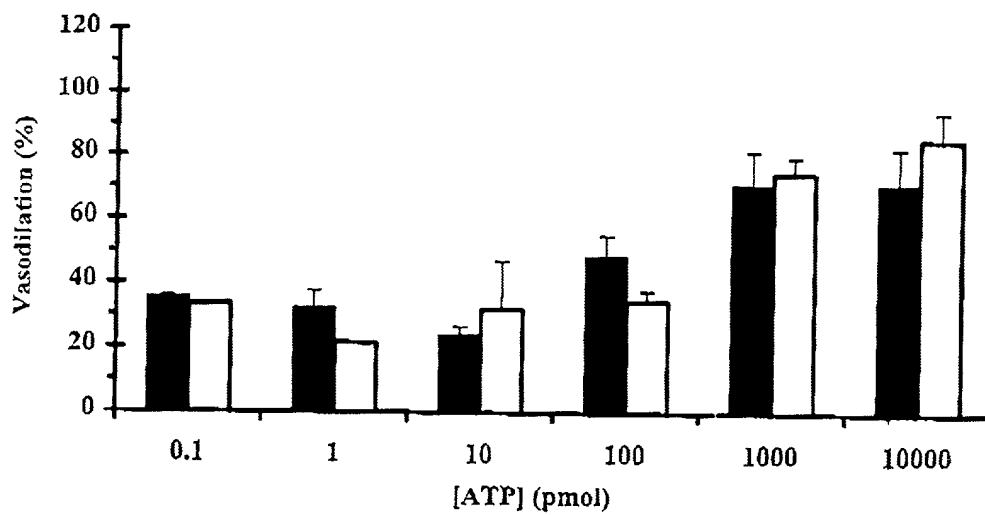


FIG. 8B

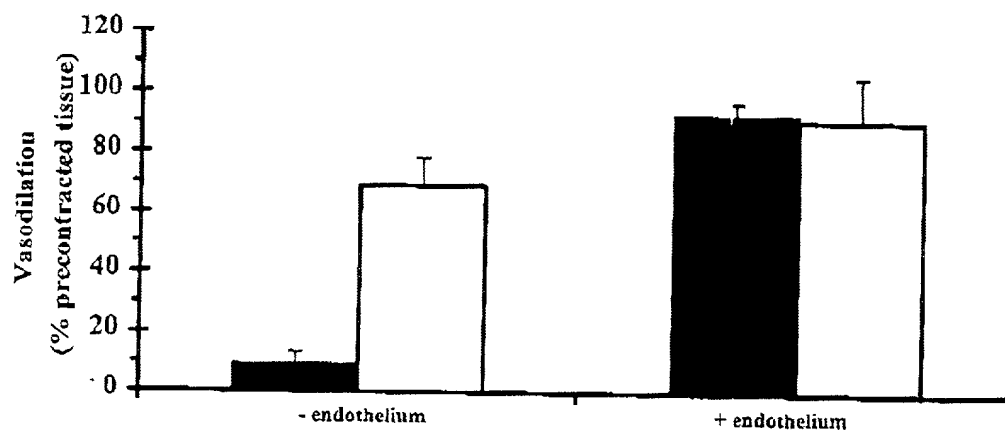


FIG. 8C

006090" 2276560

77473-10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

C-8 SUBSTITUTED PURINE NUCLEOTIDE ANALOGS AND THEIR USE AS INHIBITORS OF NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES

the specification of which

(check one) ☒ is attached hereto.

☐ was filed on _____

as U.S. Application Serial No. _____

☐ was filed on _____

as PCT International Application No. _____

and (if applicable) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §§1.56(a) and (b), which state:

- "(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:
- (1) prior art cited in search reports of a foreign patent office in a counterpart application,
 - (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

- (b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

0059477 060500 2276550

- 2 -

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability."

I hereby claim foreign priority benefits under 35 United States Code, §119 and/or §365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing of this application:

PRIOR FOREIGN APPLICATION(S)

<u>Number</u>	<u>Country</u>	<u>Filing Date</u> (<u>Day</u> / <u>Month</u> / <u>Year</u>)	<u>Date First</u> <u>Laid-open or</u> <u>Published</u>	<u>Date Patented</u> <u>or Granted</u>	<u>Priority Claimed?</u>
---------------	----------------	-------------------------------------------------------------------	--------------------------------------------------------------	-------------------------------------------	--------------------------

I hereby claim the benefit under 35 United States Code, § 119(e) of any United States provisional application(s) listed below:

<u>Application Number</u>	<u>Filing Date</u>
---------------------------	--------------------

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

PRIOR U.S. OR PCT APPLICATION(S)

<u>Application No.</u>	<u>Filing Date</u> (<u>day</u> / <u>month</u> / <u>year</u>)	<u>Status</u> (<u>pending</u> , <u>abandoned</u> , <u>granted</u>)
------------------------	-------------------------------------------------------------------	-------------------------------------------------------------------------

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

006090" 050900

- 3 -

I hereby appoint the following patent agents with full power of substitution, association and revocation to prosecute this application and/or international application and to transact all business in the Patent and Trademark Office connected therewith:

JAMES D. KOKONIS (Reg. No. 21178)
ALAN R. CAMPBELL (Reg. No. 26129)
A. DAVID MORROW (Reg. No. 28816)
JAMES MCGRAW (Reg. No. 28168)
JOHN BOCHNOVIC (Reg. No. 29229)
JOY D. MORROW (Reg. No. 30911)
TOKUO HIRAMA (Reg. No. 32551)
YWE J. LOOPER (Reg. No. 43758)
KOHJI SUZUKI (Reg. No. 44467)
R. JOHN HALEY (Reg. No. 29502)
NEIL E. HAMILTON (Reg. No. 19869)
BARRY E. SAMMONS (Reg. No. 25608)
NICHOLAS J. SEAY (Reg. No. 27386)
HARVEY D. FRIED (Reg. No. 28298)
CARL R. SCHWARTZ (Reg. No. 29437)
KEITH M. BAXTER (Reg. No. 31233)
JOSEPH W. BAIN (Reg. No. 34290)
JEAN C. BAKER (Reg. No. 35433)
BENNETT J. BERSON (Reg. No. 37094)
ALLEN J. MOSS (Reg. No. 38567)
JILL A. FAHRLANDER (Reg. No. 42518)
DANIEL G. RADLER (Reg. No. 43028)

HUGH O'GORMAN (Reg. No. 26140)
NICHOLAS H. FYFE (Reg. No. 26134)
ROBERT D. GOULD (Reg. No. 27523)
THOMAS R. KELLY (Reg. No. 29244)
MICHAEL E. WHEELER (Reg. No. 29246)
DONALD F. PHENIX (Reg. No. 32528)
R. ALLAN BRETT (Reg. No. 40476)
PHILIP D. LAPIN (Reg. No. 44443)
GRANT W. LYNDIS (Reg. No. 44484)
CHRISTINE N. GENGE (Reg. No. P-45405)
THOMAS W. EHRMANN (Reg. No. 20374)
J. RODMAN STEELE (Reg. No. 25931)
GEORGE E. HAAS (Reg. No. 27642)
MICHAEL J. MCGOVERN (Reg. No. 28326)
GREGORY A. NIELSON (Reg. No. 30577)
JOHN D. FRANZINI (Reg. No. 31356)
ROBERT J. SACCO (Reg. No. 35667)
DAVID G. RYSER (Reg. No. 36407)
MICHAEL A. JASKOLSKI (Reg. No. 37551)
SHERRY WHITNEY (Reg. No. 39422)
SCOTT D. PAUL (Reg. No. 42984)
STEVEN J. WIEI'RZNY (Reg. No. 44402)

PLEASE SEND CORRESPONDENCE TO:

QUARLES & BRADY LLP
P.O. Box 2113
Madison, Wisconsin. 53701-2113
U.S.A.
Telephone: (608) 251-5000
Facsimile: (608) 251-9166

005090" 4476560

- 4 -

1) INVENTOR'S SIGNATURE: _____ Date: _____

Inventor's Name: Adrien R. Beaudoin
(First) (Initial) (Family Name)Country of Citizenship: CanadaResidence: Rock Forest, Quebec, Canada
(City, Province, Country)Post Office Address: 748 Blvd. Des Vétérans, Rock Forest, Quebec, J1N 1Z7, Canada

2) INVENTOR'S SIGNATURE: _____ Date: _____

Inventor's Name: Fernand-Pierre Gendron
(First) (Initial) (Family Name)Country of Citizenship: CanadaResidence: Trois-Rivières, Quebec, Canada
(City, Province, Country)Post Office Address: 800 rue des Cèdres, Trois-Rivières, Quebec, G8Y 2P8, Canada

3) INVENTOR'S SIGNATURE: _____ Date: _____

Inventor's Name: Efrat Halbfinger
(First) (Initial) (Family Name)Country of Citizenship: IsraelResidence: Raanana, Israel
(City, Country)Post Office Address: 19 Hagalil St., Raanana, Israel

4) INVENTOR'S SIGNATURE: _____ Date: _____

Inventor's Name: Bilha Fischer
(First) (Initial) (Family Name)Country of Citizenship: IsraelResidence: Shoham, Israel
(City, Country)Post Office Address: 75 Aadulam St., Shoham, Israel

000000 " 22765500